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WHEAT GERM AGGLUTININ BINDING SITES ON THE CELL SURFACES OF THE EARLY CHICK EMBRYO

by

© A. ROBERT ANDERSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled WHEAT GERM AGGLUTININ BINDING SITES ON THE CELL SURFACES OF THE EARLY CHICK EMBRYO submitted by A. ROBERT ANDERSON in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

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Date April 20, 1978

ABSTRACT

The cell surface has been connected with development and embryogenesis along with other cellular events. To study the relationship between the cell surface components and gastrulation, the distribution of Wheat Germ Agglutinin (WGA) binding sites was determined on Stage 1 and Stage 5 (Hamburger and Hamilton, 1951) in the chick embryo. WGA binds specifically to terminal n-acetyl-glucosamine (GlcNAc) residues and is localized by the enzyme markers horseradish peroxidase (HRP) and glucose oxidase (GO). These marker enzymes react with diaminobenzidine (DAB) to form an electron dense reaction product which is visualized in the electron microscope. In addition, attempts were made to tag WGA with the electron dense marker ferritin.

The results were inconclusive for all the labelling techniques except the enzyme marker GO. The distribution of WGA binding sites, and hence terminal GlcNAc residues as revealed with GO activity, is altered from the Stage 1 to the Stage 5 embryo. In the Stage 1 embryo, the WGA binding sites have a relatively even distribution on all surfaces. However, in the Stage 5 embryo, the WGA binding

sites are segregated to the cell surfaces that line the cavity between the epiblast and endoblast. Furthermore, the mesenchyme cells that are invaginating through the primitive streak have a very low affinity for WGA, but as they migrate away from the streak they regain their WGA binding sites.

It appears that the WGA binding sites, and consequently the terminal GlcNAc residues, may be directly involved in the process of gastrulation and morphogenesis in the early development of the chick embryo. Although the molecular mechanisms controlling the polarity of the WGA binding sites on specific cell surfaces are not understood, they may have a direct influence upon the development of the chick embryo.

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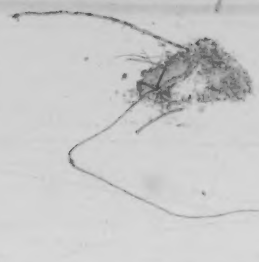


93-94 Schematic representation of the WGA binding sites on the cell surfaces of the Stage 1 and Stage 5 chick embryo as revealed with GO. 129-131

LIST OF ABBREVIATIONS

WGA	Wheat Germ Agglutinin
GlcNAc	n-acetyl-glucosamine
HRP	Horseradish peroxidase
Fe	ferritin
GO	glucose oxidase
DAB	3'3' diaminobenzidine tetrahydrochloride
DE	dorsal surface of the epiblast
LE	lateral surface of the epiblast
VE	ventral surface of the epiblast
DH	dorsal surface of the hypoblast
VH	ventral surface of the hypoblast
DM	dorsal surface of the mesenchyme
VM	ventral surface of the mesenchyme
DN	dorsal surface of the endoblast
VN	ventral surface of the endoblast
glut	glutaraldehyde
d $H_2Ø$	distilled water

INTRODUCTION

Cells, in general, have complex oligosaccharides extending from their cell surface that appear to form a coat around them (Martinez-Palomo, 1970). These carbohydrate chains seem to be specifically synthesized upon protein backbones or lipid molecules (Law and Snyder, 1972; Bennett et al., 1974). The majority of the evidence supports the theory that the carbohydrate portion is added sequentially by glycosyltransferases in the Golgi apparatus and ultimately is deposited on the external surface of the cell (Singer and Nicolson, 1972; Roth, 1973). It has been shown that these complex oligosaccharides may form the basis for intricate cellular events such as adhesion, recognition, communication, migration and growth regulation (Cook and Stoddart, 1973; Roseman, 1975; Poste and Nicolson, 1976b). In addition, the cell surface has been implicated to be involved with development and embryogenesis (reviewed by Moscona, 1974, Poste and Nicolson, 1976b). In the present study, the cell surface carbohydrates were examined in relation to the early development of the chick embryo.

One of the techniques of studying the cell surface is the utilization of the molecules collectively known as lectins (reviewed by Nicolson, 1974a). These proteins and glycoproteins have the property of binding to specific sugar residues upon the cell surface. The purpose of this study was to utilize the lectin, wheat germ agglutinin (WGA), to determine the relationship between terminal n-acetyl-glucosamine (GlcNAc) residues and the development of the chick embryo from Stage 1 to Stage 5 (Hamburger and Hamilton, 1951). The distribution of WGA binding sites, and hence terminal GlcNAc residues, was revealed in the electron microscope by the enzyme markers horseradish peroxidase (HRP; Francois et al, 1972; Huet and Garrido, 1973) and glucose oxidase (GO; Francois and Mongiat, 1977) and their subsequent histochemical reaction with diaminobenzidine (DAB). In addition, WGA was also tagged with the electron dense marker ferritin and visualized upon the cell surface with the electron microscope (Nicolson et al, 1975; Shimizu and Yamada, 1976).

The WGA was incubated with chick embryos that were either fixed or unfixed. Then the WGA binding sites were histochemically stained and the embryos prepared for electron microscopy. The results indicated that from Stage

1 to Stage 5, in the chick embryo, the distribution of terminal GlcNAc residues, as revealed by WGA binding sites, was altered. The WGA binding sites on the Stage 1 embryo had a relatively even distribution upon all the cell surfaces. However, the Stage 5 embryo displayed WGA binding sites that appeared to be limited to specific cellular surfaces. These distributions seemed to be fairly similar throughout the embryo, and on the Stage 5 embryo the terminal GlcNAc residues had an interesting pattern. There appeared to be a high concentration of GlcNAc residues lining the cavity between the epiblast and the hypoblast. The mesenchyme cells apparently lost their terminal GlcNAc residues as they passed through the primitive streak, but retained their WGA binding sites as they migrated away from the streak. Finally, the lateral surfaces of the epiblast in the Stage 5 embryo did not have any exposed GlcNAc residues. These results demonstrated that by the time of gastrulation within the chick embryo, there had been cell surface differentiation in relation to development.

The present study has shown that the distribution of WGA binding sites is altered from Stage 1 to Stage 5. The molecular mechanism for this reorganization is unknown and experiments should be conducted to understand this

process. Furthermore, the actual importance of the terminal GlcNAc residues in the process of gastrulation should be explored.

LITERATURE REVIEW

In 1972, Singer and Nicolson, utilizing their basic understanding of the physical chemistry of lipid and protein interactions, formulated the Fluid Mosaic Model for biological membranes. In this model, the components of membranes (proteins, lipids and carbohydrates) were depicted as a fluid lipid bilayer with islands of proteins. The carbohydrate portions of the glycolipids and glycoproteins were exposed to the external medium. Some proteins were firmly embedded in the lipid bilayer and others extended through the bilayer (intrinsic proteins). The remaining membrane proteins were loosely associated with the internal or external surface (extrinsic proteins). This model, formulated largely upon research conducted on the erythrocyte membrane (reviewed by Bretcher, 1971a, b; Steck, 1974; Marchesi et al, 1976), has been widely accepted.

1. Glycoprotein Structure:

The surfaces of eukaryotic cells have been shown to contain a large amount of carbohydrates (Martinez-Palomo, 1970; Winzler, 1970; Parsons and Subjeck, 1972; Cook and

Stoddart, 1973; Ito, 1974) located on glycoprotein or glycolipid molecules and as glycosaminoglycans. These carbohydrate-containing moities have been shown to turn over rapidly during cell growth (Warren and Glick, 1968; Warren, 1969; Rosenburg and Einstein, 1972; Fritsch et al, 1975; Buck and Warren, 1976). Glycoproteins and glycolipids have been demonstrated to act as receptors for: histocompatibility antibodies (Cunningham, 1977); hormones such as insulin (Narahara, 1972); blood group antibodies (Marchesi et al, 1976); immune reactions (Raff, 1977); and lectin-mediated agglutination (Sharon, 1977) and mitogenesis (Nicolson, 1974a). The extracellular glycosaminoglycans are of paramount importance in the formation of connective tissue and have been implicated as a substrate for cell migration (Solursh, 1976).

The structures and sequences in the carbohydrate portion of the major glycoproteins have been determined by systematically degrading the molecule with selective enzymes (for reviews see Kornfeld and Kornfeld, 1976; Marchesi et al, 1976). This type of analysis involves degrading the glycoprotein molecule with proteases, fractionating the products and selectively hydrolyzing the carbohydrates with exoglycosidases or endoglycosidases. When analysis of this

type is attempted, the need for highly specific enzymes is imperative, due to the large number of variable structures that may exist within the carbohydrate chain of the glycoprotein. The sugar molecules that are present in these macromolecules have the capability of linking to each other at various free hydroxyl groups, with the result that in a hexose dissaccharide it is possible to have thirty-six combinations. The complexity that may exist in the sequence of straight, and more often, branched oligosaccharides is considerable, yet is probably fundamental to their function.

The glycosylation of glycoproteins and glycolipids occurs by two mechanisms. Although the majority of this process occurs in the Golgi apparatus, some glycosylation occurs in the endoplasmic reticulum and upon the plasma membrane (Bennett et al, 1974). Glycolipids are synthesized from a diglyceride or ceramide with the transfer of a sugar molecule from a nucleotide to the free primary hydroxyl group at the three or one position respectively. The nucleotide carrier may also add the sugar to the growing oligosaccharide chain (Law and Snyder, 1972). Glycoproteins are synthesized by the addition of a phosphorylated sugar molecule to one of the amino acids; serine, threonine or asparagine. The nucleotide sugar carrier, or in the case of

core N-acetyl-glucosamine or mannose residues of asparagine-linked side chains, the dolichol phosphate lipid carriers, donate their specific sugars to the appropriate amino acid with a glycosyltransferase specifically creating the reaction (reviewed by Waechter and Lennarz, 1976). Asparagine-linked side chains are started in the endoplasmic reticulum even before the whole protein has been synthesized, but the terminal sugars are added in the Golgi apparatus. Less is known about where the core sugar, N-acetyl-glucosamine is added to the serine or threonine residues (Schacter, 1974). The specificity of the addition of the sugar moities to the growing oligosaccharide is almost completely understood and seems to involve both the protein backbone and the oligosaccharide sequence (Roseman, 1970, 1975; Cook and Stoddart, 1973; Roth, 1973; Leblond and Bennett, 1977).

It has been proposed that glycosyltransferases are present in the serum of animals and on a variety of cell surfaces (Deppert et al, 1973; Pratt and Grimes, 1973), although this theory has been disputed (for review see Keenan and Morre, 1975). The possibility of glycosyltransferases on the cell surface may be very significant, for it provides a specific mechanism to explain cell-specific

aggregation and sorting out, or such surface-associated events as adhesion, agglutination and morphogenesis (Roth, 1973).

2. Glycoproteins as Cell Surface Receptors:

Membrane associated glycoproteins probably act as receptors, with the specificity either in the carbohydrate chain or in the amino acid sequence. They have been demonstrated to be highly mobile within the plane of the membrane (Frye and Edidin, 1970) and to diffuse rapidly over the cell surface. This diffusion will be discussed further in relation to the cytoplasmic influence over surface mobility. Some of the glycoproteins and proteins have been shown to pass through the bilayer and to intimately associate with the hydrophobic portions of the lipids but are yet highly mobile. In considering the erythrocyte membrane or the plasmalemma of the organism Acholiplasma laidlawii evidence has been presented that the particles seen with freeze fracture (Pinto da Silva and Branton, 1970; Branton, 1971; Marchesi, 1973; Singer, 1973) are aggregates of glycoprotein molecules. With ferritin labelling of the glycoproteins, in conjunction with freeze fracturing, it was hypothesized that these particles were

glycoproteins that could exist in a dispersed or clustered state in the membrane. The studies on A. ladlawii showed that at the optimal growth temperature the particles were in a dispersed condition in the membrane. However, when the temperature was lowered, the particles became clustered and growth was diminished (James and Branton, 1973). After further study it was concluded that proteins must remain in the fluid environment of the lipid bilayer for optimal activity. That is, the proteins would be squeezed out of lipid domains which are in a gel state and remain, preferably, in the domains of lipids that would be in the fluid (liquid crystalline) state (De Kruyff et al, 1973).

As mentioned above, Frye and Edidin (1970) discovered that there was rapid intermixing of cell surface antigens and that these surface glycoproteins were extremely mobile within the plane of the membrane. With the evidence that these antigens may span the bilayer and the finding that antibody binding to surface receptors would produce a distribution that was either diffuse, clustered, or capped (Taylor et al, 1971), there was intense study of the mechanisms that control the surface distribution of the glycoproteins. Lymphocytes were the major target of investigation, and when the surface antigens were capped

(glycoproteins orientated at the uropod over the Golgi), the receptors were subsequently pinocytosed and the lymphocyte was transformed mitogenically. However, cytochalasin B (considered to disrupt cytoplasmic microfilaments, inter alia) inhibited capping to varying degrees, but did not alter the initial patching response (reviewed by Nicolson, 1976a). Therefore, the distribution of surface receptors was found to be related to the microfilament cytoskeleton of the lymphocyte.

Further study of the cytoplasmic control over the distribution of the surface receptors revealed: that capping is energy and temperature dependent; that by cross-linking receptors, other antigens are unable to cap; that microtubules act as anchors, and microfilaments must be present to induce capping; and that Ca++ release and local anesthetics induce capping (for excellent reviews see Edelman, 1976, and Nicolson, 1976a). Fuller and Brinkley (1976) hypothesized that the initial step in the modulation of the distribution of the surface antigens by the cytoplasmic microtubule complex is the release of Ca++. This was supported by the experiments of Poste and Nicolson (1976a), who found that ionophores that specifically transport Ca++ mimicked the effect of colchicine or other

microtubule disrupting drugs. Although it appears that microfilaments are the driving force in the capping phenomenon, it was found that in certain cell lines cytochalasin B causes microfilament disruption and capping, which could be reversed by the addition of colchicine (Sundquist and Ehrnst, 1976; Williams et al, 1977). This appears to be evidence in support of the fact that either microfilaments or microtubules may by themselves induce capping, but with both absent no such response may be initiated. Although no definite common mechanism is discernible, Edelman et al, (1973) have proposed a hypothesis for the relationship between surface receptors and their cytoplasmic components. The theory is that the surface antigens are linked to microfilaments which interact with microtubules; by disrupting the microtubules the Surface Modulating Assembly will cause the antigens to cap. However, by cross-linking certain receptors, the remainder of the antigens may not spontaneously cap unless the anchoring microtubules are disrupted (for review see Edelman, 1976).

In 1977, Wolpert suggested that the plasma membrane must function as a mechanical sensor, a channel, and a transducer in such cellular interations as adhesion, recog-

nition, communication and differentiation. The most probable components of the membrane which would have the capacity to mediate such events are glycoproteins since they may completely span the membrane. Some examples will be considered below.

(a) Moscona (1952, 1961) introduced standard methods for dissociation and developed the rotary shaker technique for aggregation studies. He then initiated the development of the ligand hypothesis for cell recognition and selective cell affinities (for review see

Moscona, 1974). His group found that dissociated cells secrete a tissue-specific aggregating factor. This ligand binds specifically to receptors present on the cell surface and enhances the adhesion and aggregation of homotypic cells in vitro. They also reported the presence of tissue-specific antigens on embryonic cell surfaces. The ligand hypothesis postulates that sorting out is mediated by specific cell surface antigens that act in recognition and morphogenesis (Hausman and Moscona, 1976). This group has, in fact, isolated a glycoprotein that has the specific effect of enhancing the aggregation of chick neural retinal cells, from which it came.

Roth (1973) proposed that recognition and adhesion are possibly due to glycosyltransferases on the surfaces of cells. His theory would suggest that like cells would have similar glycosyltransferases and receptors, so that more stable associations would be formed in homotypic cell types. The

enzyme would form a bridge between cells that had on their surfaces glycoproteins or glycolipids with sugar sequences that the glycosyltransferase could recognize as a suitable substrate. These bonds would be relatively short-lived if there were any nucleotide sugar carriers present, but could bring the cells together long enough to form stable contacts and sort out.

The recognition and subsequent adhesion between homotypic groups of cells has also been related to terminal sialic acid residues. Lloyd (1973) suggested that sialic acid could function as a recognition site protector in such a manner as to mask the receptor function. He hypothesized that adhesion between cells would depend upon the appropriate conformation of the receptors to suit the bonds to be formed. In addition, the presence of sialic acid on the cell surface would tend to increase the cell's net negative charge, causing the repulsion of some cells more forcefully than others.

(b) The process of recognition has been studied in the immunological systems of animals. A variety of antigens on cells allow the lymphocytes to recognize the cells as alien or native. The recognition process is determined by appropriate antibody formation resulting from any new antigen that is introduced to the system. When the antibodies recognize an antigen they bind to it and it is subsequently removed from the system (for reviews see Raff 1976; Cunningham, 1977). The presence of specific antibodies within the animal provides protection against any further antigenic invasion.

(c) Malignant transformation has been related to a dedifferentiated state of the cell. The cell loses its antigenicity and adhesion properties, begins to divide without any apparent control, and may even invade healthy organs, causing them to malfunction. Alterations appear to occur in the cell surface which may reflect malignant transformation. It has been suggested that transformed cells have an

altered surface modulating assembly, a higher susceptibility to agglutination by phytohem-agglutinins, and a loss of a high molecular weight glycoprotein related to lowered adhesion and uncontrolled cell cycle properties (reviewed by Abercrombie, 1967; Dunn 1971; Nicolson, 1976, a and b).

3. Early Chick Embryology and the Cell Surface:

At the time of laying the chick embryo appears as a circular disc composed of about 60,000 cells (Spratt, 1963). There are two distinct areas within the blastoderm: the central area pellucida and the peripheral area opaca. The area pellucida is generally composed of a dorsal layer of columnar cells called the epiblast and a ventral layer of larger spheroid cells called the hypoblast. The area opaca is composed of five or six cell layers (Bellairs, 1971). Small yolk granules are formed in close association with the area opaca while large white yolk granules appear in loose association with the area opaca, and the area pellucida. Beneath the area

pellucida is a cavity called the subgerminal space which is filled with embryonic fluid (Pasteels, 1945).

The area pellucida will form the future embryo while the cells of the area opaca will give rise to extra-embryonic tissue. When the egg leaves the uterus, the area pellucida may have a second layer of cells beginning to form beneath the epiblast. These cells, the primary hypoblast, appear to come from the epiblast via a process of delamination (Peter, 1938). The primary hypoblast first appear as islands of cells beneath the epiblast. With further incubation the primary hypoblast will aggregate, form the embryonic shield or "sickle of Koeller" (a disc of cells at the posterior end of the area pellucida), and then move anteriorly and laterally as a sheet of cells beneath the epiblast (Eyal-Giladi and Kochav, 1976). It appears that when the egg leaves the uterus the degree of development of the primary hypoblast is variable; that is, if the egg is held within the uterus for a slightly longer time, the primary hypoblast may be starting its anterior migration.

In 1951, Hamburger and Hamilton depicted the unincubated embryo as a circular disc with few or no distinguishing features. This embryo was called Stage 1. The Stage 2 embryo showed the initial formation of the primitive streak. Eyal-Giladi and Kochav (1976) removed eggs artificially from the uterus of the hen and divided Hamburger and Hamilton's first two stages into fourteen. They described the early movements within the embryo in great detail and explained the development between the unincubated and early primitive streak chick embryo.

As the primary hypoblast is forming a continuous layer beneath the epiblast, the epiblast cells are beginning to prepare for gastrulation. It is known that the hypoblast orientates the future embryonic axis (Waddington, 1933, 1956; Eyal-Giladi, 1970) and may influence the epiblast to begin to invaginate through the central region of the area pellucida called the primitive streak. Bancroft and Bellairs (1974) showed with scanning electron microscopy that cells which are about to move into the primitive streak region have less microvilli and more globular projections than the peripheral cells that

will form the ectodermal layer of the embryo. They also noted cellular projections, which they term cords, extending from one cell over to another cell, possibly keeping the cells in communication with each other.

The morphogenic movements of cells within the chick embryo have been studied by cinephotomicrographic observation (Vakaet, 1970) and have been mapped with a wide variety of dyes or labels (Spratt and Haas, 1960; Rosenquist, 1971, 1972). The consensus of opinion is that the initial group of cells to invaginate through the primitive streak form a layer of cells (secondary hypoblast or endoblast) which invade the primary hypoblast and push the original layer out to the periphery, where it begins to form the yolk sac membrane (Bellairs, 1963; Vakaet, 1970; Rosenquist, 1971). The endoblast forms a continuous sheet of cells which underlies the epiblast and will form the future endoderm. The cells that follow the endoblast through the primitive streak will form the mesoderm of the future embryo.

In the early development of the chick embryo from Stage 1 to Stage 5, some cell surface alteration

is perceptible. Apparently, the electrophoretic mobility at pH 7.2 of dissociated embryos decreases from unincubated to Stage 5 (Zalik et al, 1972). Furthermore, Zalik et al found that sialic acid contributed little to the surface net charge. Also, there is an associated decrease in the amount of glycoprotein present at the cell surface as the embryo began to form the primitive streak, as revealed with lanthanum-nitrate and colloidal iron binding (Sanders and Zalik, 1972). In addition, Sanders and Zalik (1972) found that there was a large amount of lanthanum staining present in junctional areas of the chick blastoderms and within the primitive streak region. This indicated an increased amount of surface carbohydrate moities in these two regions.

In sorting out experiments, the hypoblast was found to have altered surface properties from the epiblast. In 1974, Zalik and Sanders showed that the primary hypoblast would sort out from aggregates of dissociated unincubated embryos. Also, in 1975, Eyal-Giladi et al showed that the primary hypoblast would sort out and migrate to the edges of aggregates of unincubated embryos with the vitelline membrane as a

substrate. The aggregate would form a distinct two-tiered structure, with the larger, heavier primary hypoblast cells positioned ventrally and the epiblast cells dorsally. Bellairs et al (1977) found that when cultures of explanted endoblast and primary hypoblast cells confront one another, the former invades the latter and eventually the primary hypoblast surrounds the endoblast. In addition, the primary hypoblast has been shown to have an increased number of Concanavalin A binding sites on its ventral surfaces, as compared to its dorsal surfaces (Hook and Sanders, 1977) indicating cell surface specialization reflecting cell differentiation.

4. The use of Lectins in Probing the Cell Surface:

A. Agglutination Studies:

The history and development of the use of lectins as probes of the cell surface have been reviewed extensively by Sharon and Lis (1973, 1976), Nicolson (1974a), and Rapin and Burger (1973). Lectins (or phytohemagglutinins) are plant or animal

proteins that cause agglutination of red blood cells. These proteins, which include Concanavalin A (Con A), Wheat Germ Agglutinin (WGA), Ricin Communis Agglutin (RCA) or Soyabean Agglutinin (SBA), bind specifically to carbohydrate moities at the cell surface. For example, the WGA-induced agglutination of erythrocytes, type A, is specifically inhibited by N-acetyl-glucosamine (GlcNAc). This indicates that WGA binds to terminal GlcNAc residues on the cell surface. Con A is specific for terminal α-methyl mannoside-like residues at the cell surface because agglutination can be inhibited by the presence of α-methyl mannoside in the incubation medium.

Agglutination has been interpreted as the cross-linking of like receptors on two cell surfaces by a multivalent ligand. By studying the patterns of agglutination caused by a series of lectins, the investigator may determine the presence or absence of specific carbohydrates at the cell surface. However, two conditions should be met before agglutination will occur. Firstly, the lectin must be multivalent, and secondly, the receptors should be mobile on the cell surface. For instance, succinyl Con A, a univalent

lectin, will not agglutinate or initiate movement of the surface receptors to the extent that the tetravalent Con A does (Gunther et al., 1973). The mobility of the receptors has been recognized to be important through experiments which showed that although equal amounts of lectin bind to glutaraldehyde-fixed cells versus non-fixed cells, the non-fixed cells are agglutinable by much lower dosages of the lectin than the fixed cells. This indicates that a loss of the receptor mobility decreases agglutination, but this can be overcome by increasing the number of cross-linking lectins mediating agglutination (Inbar et al., 1973; Noonan and Burger, 1973). Therefore, by noting the variation in agglutination, not only can the surface topography be characterized, but the conditions of the membrane receptor mobility could be interpreted also.

For quantitative probing of the cell surfaces, lectins have been tagged with radioactive markers. This allows comparisons in the binding of a variety of cell types and conditions. The investigator may quantify the number of lectin binding sites and cross reference them to agglutination studies for surface

mobility. The most useful technique involves lactoperoxidase (Arndt-Jovin and Berg, 1971) coupling of ^{125}I to the lectin and subsequent analysis of binding (Ozanne and Sambrook, 1971).

Lectins such as Con A and WGA have been found to be useful in the study of surface conditions of transformed cells (for review see Noonan and Burger, 1974). It has been recognized that non-transformed cell lines are agglutinated only after trypsinization, whereas transformed cells can be readily agglutinated by lectins. Aub (1963) was one of the first investigators to discern that the difference between transformed cells and normal cells may lie at their cell surfaces. His hypothesis seems well founded for the characteristic conditions of cancer cells, that is, contact inhibition loss, rapid division, altered morphology and lowered adhesion would seem to indicate some altered membrane structure. There appears to be a loss of glycoproteins from the transformed cell membrane as detected with lactoperoxidase iodination (Pouyssegur and Pastan, 1976). The ability to probe the cell surface of transformed cells will make possible a potentially large amount of information with

regard to the transformed state and may eventually allow for methods of controlling the transformed state. The use of lectins and other such probes will aid this research.

Lectins also stimulate mitogenesis (Lis and Sharon, 1973) and are therefore useful in the study of lymphocyte activation. Apparently the most important factors in mitogenesis stimulation are the valency of the lectin (Mannino and Burger, 1975) and the capping response of the lymphocyte.

B. Ultrastructural Visualization of Lectin Sites:

The sites where the lectins have bound to the cell surface may be visualized and quantified by a variety of techniques. At the level of the light microscope, the lectins may be tagged with radio-isotopes and studied by autoradiography (as mentioned above), fluorescein isothiocyanate (Smith and Hollers, 1970), or tetramethylrhodamine isothiocyanate (Loor, 1973). The fluorescent tagging method is very useful

since the dynamics of the lectin receptors may be observed on living cells. This was the method used to discover the patching and capping responses of lymphocytes (Taylor et al., 1971; Karnovsky et al., 1972; Yahara and Edelman, (1972).

Cell surface bound lectins may be located in the electron microscope by a variety of techniques. The most often used is the conjugation of the lectin via glutaraldehyde to an enzyme marker, such as horseradish peroxidase (HRP, Avrameas, 1969) which is electron dense after a reaction with diaminobenzidine (DAB, Graham and Karnovsky, 1969), or to the electron dense marker Ferritin (Nicolson and Singer, 1971). Recently, the lectin, Con A, was located on the cell surface with the specific marker Busycon canaliculatum hemocyanin (Smith and Revel, 1972). This marker has a distinct size and shape which aids in its recognition on the cell surface by the use of replica techniques. These three techniques were compared in a study performed by Temmink et al (1975). They speculated that the limitations of these techniques were due to the marker size, the inability to determine the effect of conjugation upon the binding ability of the lectin,

and the enzymatic reaction that takes place between the marker HRP and DAB. They suggested that *in vitro* conjugation of the lectin to the marker was a better technique because of its more direct binding and visualization. However, they noted that any enzymatic reaction could produce an overestimation of the number of lectin receptors present. The ferritin or hemocyanin markers were considered to be the most accurate in determining the number of lectin binding sites, but the inaccessibility of some of the surface sites to these labels is a limitation.

C. Lectins and Development

With the development of lectins as specific probes for the surface characteristics, much attention was devoted to changes in cell surfaces in relation to cellular differentiation. In 1972, Kleinschuster and Moscona found that in association with differentiation there was an altered ability for Con A to induce agglutination. They found that as retina cells from eight- or nine-day old chick embryos differentiate and mature, they lose the property of agglutinability with

Con A. However, by treating thirteen-to sixteen-day old retinal cells with trypsin first, the Con A induced agglutination. Then, in 1973, Krach et al found a similar trend for developing sea urchin embryos. They found that as the sea urchin embryo developed from one to two and three days old, there was a decrease in the agglutination with Con A or RCA. Interestingly, they found that WGA had no effect upon agglutination in sea urchin embryo cells until they were trypsinized. These developmental changes may be associated with a decrease in the fluidity of the membrane (Martinozzi and Moscona, 1975). Roquet et al (1976) found that during development there was an altered fibroblast response to a variety of mitogenic lectins. By monitoring the amount of (^{3}H) thymidine incorporation after incubation of chick embryo fibroblasts (eight- to sixteen-day) with Robino lectin, Dolichos lectin or Con A, they found an increase in mitogenesic response of the fibroblasts from the sixteen-day embryo to the lectins. There was a definite gradient of mitogenic response, although the total amount of lectin binding sites decreased with development. Experiments from the same lab showed that there was an associated decrease in agglutination of fibroblasts from the

eighth to sixteenth days of development (Aubery and Bourrillon, 1976).

Zalik and Cook (1976) found that there was an associated loss of WGA-induced agglutination of chick embryo cells with development. They found that Stage 1 to Stage 5 chick embryo cells were agglutinated by WGA, Con A and RCA but not by fucose-binding protein, and that SBA would induce agglutination only after neuraminidase treatment. However, trypsin pre-treatment of twelve-day liver cells would induce WGA agglutination but had no effect upon blastoderm cells. They also found that the trypsinized twelve-day liver cells bound less WGA than EDTA-dissociated cells and that the dissociated blastoderm cells bound similar amounts of WGA as the twelve-day cells. They thought that WGA receptors were masked with trypsin-sensitive material as development proceeded, indicating that the WGA receptors were altered with differentiation.

As well, further studies with Con A have implicated these receptors in early development and differentiation. In Xenopus embryos, O'Dell et al (1974) showed that fluorescein-labelled Con A had a

higher affinity for the dorsal lip of gastrulating embryos and the presumptive neural plate during neurulation. In sea urchin embryos, Oppenheimer's group found that micromeres were preferentially agglutinated by Con A (Neri et al, 1975; Roberson et al, 1975). They also showed that fluorescein Con A had a greater lateral mobility on the micromere cell surface than on the mesomere or macromere cell membranes.

The decrease in agglutination as a function of differentiation is probably not related to a decrease in the fluidity of the bilayer, because agglutination cannot be related to fluidity (Gaffney, 1973; Lyles and Landsberger, 1976; Bales et al, 1977). Also, the surface characteristics of cells, that is, the presence or absence of microvilli, have been shown to have no correlation with agglutinability (Ukena and Karnovsky, 1977). Finally, the decrease in agglutination cannot be attributed to a decrease in the number of lectin binding sites, as the amount of lectin binding remains the same or increases as development proceeds. The alteration that has apparently not been examined is the relationship of cytoplasmic control over the surface

receptors and the effect of trypsin upon the surface modulating assembly. Although masking of surface receptors is a very probable method in differentiation, the cytoplasmic control should be considered as well.

D. Wheat Germ Agglutinin:

Wheat Germ lipase was found to agglutinate malignant cells specifically (Aub et al, 1963) and the actual Wheat Germ Agglutinin (WGA) was purified by Burger (1969). The molecule was originally thought to be a glycoprotein (Nagata and Burger, 1972); however, it has been determined with further purification that WGA is a protein rich in sulphur-to-sulphur bonds (as well as half-cystine, glycine residues; LeVine et al, 1972, Allen et al, 1973). The binding site is similar to that of hen's egg-white lysozyme and is like a cleft in the molecule. From binding studies it appears that the binding affinity is much greater for oligomers of N-acetyl-glucosamine (GlcNAc), so that chitobiose $(GlcNAc)_2$ has a higher affinity than GlNAc at the site (Privat et al, 1974; Goldstein et al, 1975; Privat and Monsigny, 1975). WGA was also found to bind non-

specifically to Sialic acid by Greenaway and LeVine (1973). Rice (1976) found that the crude Wheat Germ from a variety of strains possesses a variety of agglutinins. He concluded that it is necessary to ensure that the source of the agglutinin is noted due to the varying isolectins that were found. Also, the method for purification should be standardized so that all laboratories would receive identical agglutinins (Wang et al, 1975). Recently, Sharon (1977) has suggested that WGA has a complex molecular configuration; that is, there are two asymmetric units per subunit weighing 23,000 daltons and two subunits with four binding sites per molecule weighing 36,000 daltons.

Wheat Germ Agglutinin has been visualized on the surface of a wide variety of cells by coupling via the glutaraldehyde method of Avrameas (1963) to horseradish peroxidase (Huet and Garrido, 1972; Gonatas and Avrameas, 1973; Garrido et al, 1974) or ferritin (Nicolson et al, 1975; Shimizu and Yamada, 1976). Recently, WGA was identified ultrastructurally with the marker enzyme glucose oxidase (Francois and Mongiat, 1977). By employing these ultrastructural techniques

of WGA visualization, these authors have found that transformation does not alter the surface binding pattern of WGA. There is, however, a general agreement that transformation causes an increased mobility of surface WGA receptors. These findings, and the evidence that WGA specifically agglutinates transformed cells over non-transformed cells, could indicate either increased membrane fluidity or disruption of the Surface Modulating Assembly with transformation. Garrido (1975) found that cells that where entering mitosis showed an increased clustering of WGA sites, as visualized by horseradish peroxidase, over cells that were in the interphase. They also reported that the mobility of surface receptor sites was not dependent upon whether the cells were in suspension or in monolayers. Furthermore, there was no correlation between colcemid treatment and mobility. This may suggest that the transformed state and the mitotic state are synonymous, with respect to the Surface Modulating Assembly.

The activity of the surface receptors seems to be similar to the Con A receptors. When the clustering of WGA receptors occurs, the distribution of other

lectin receptors is clustered also (Shimizu and Yamada, 1976). However, it could not be determined if different glycoproteins were being labelled. They did note that when cells were agglutinated with ferritin labelled lectins, that the ferritin label showed a clustered distribution on the free surfaces and in areas where the two cells were in contact. This finding supports previous information that agglutination is a cross-linked process (Nicolson, 1972).

Very few studies have been directed towards visualization of WGA receptors related to embryonic differentiation, although the evidence suggests that WGA receptors are altered with differentiation by a trypsin-sensitive material (Moscona, 1971, 1974; Kleinschuster and Moscona, 1972; Zalik and Cook, 1976). These studies have used exclusively agglutination assays to determine alteration of cell surface with differentiation. The present study was conducted to examine the relationship of WGA binding sites and the early development of the chick embryo <u>in situ</u>.

MATERIALS AND METHODS

1. PREPARATION OF CHICK EMBRYOS

Fertilized eggs from Leghorn chickens were obtained from the Poultry Farm, University of Alberta. The embryos were dissected out and the adhering yolk, the embryo and the square of vitelline membrane was scooped out and placed in cold Pannett and Compton's saline (Pannett and Compton, 1924).

Pannett and Compton's saline (PCS), a buffered isotonic saline, was prepared by mixing 47.4 g NaCl, 6.2 g KCl, 3.1 g $CaCl_2$ 5.1 g $MgCl_2$ .6 H_2O and 2.42 g Tris up to 1 litre of dH_2O. This stock solution was adjusted to a pH of 7.5 at 25°C with 1 M solution of Tris (reagent grade) and was stored in the refrigerator at 4°C until used. Before the embryos were placed into the saline, the stock solution was diluted ten times.

The embryos were then cleared of all adhering yolk granules and the vitelline membrane. They were

then transferred into separate watchglasses containing fresh PCS and placed in the refrigerator (4°C) overnight.

2. PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY

All stock solutions were routinely checked for proper pH. Those that were not in stock were made fresh before the experiment was started. The following procedure was the standard preparation. However, depending upon the conditions to be tested, these methods were altered to suit the experiment.

The embryos were removed from the refrigerator and rinsed twice for 10 minutes with 0.1 M Na phosphate buffer, (Sorenson's) pH 7.4 (the buffer). The embryos were kept cold by either rinsing with cold buffer or by placing the watchglass on ice. After rinsing, the embryos were fixed with 2.5% glutaraldehyde (from 70% stock of Ladd Research Chemicals) and in the buffer for 2 hours at 4°C. The embryos were rinsed again with the buffer three times for 10 minutes each. Embryos were then post-fixed with 1% OsO_4 in the buffer for 1 hour at 4°C. This was followed by three rinses for 10 minutes with the buffer or an overnight rinse with the

buffer at 4°C. The embryos were then dehydrated in a graded series of ethanol (10 minute rinses in 50%, 70%, 80%, 95% and two rinses in absolute ethanol). After dehydration, the embryos were immersed in propylene oxide for 30 minutes at room temperature. Then the embryos were transferred into new vials containing 1:1 propylene oxide:-araldite (Luft, 1961). After being allowed to stand overnight, the mixture was drained off and fresh araldite (about 1 ml/embryo) was layered on top of the embryo. The vials were then placed on a multi-purpose rotor (Scientific Industries Inc.) and rotated for 6 to 8 hours at room temperature.

The embryos were then scooped out of the vials and placed on a half-block of araldite, labelled and covered with fresh araldite. The rubber mold was then transferred to the oven (temperature 60°F or 15°C) and polymerized for about 60 hours. The blocks were then removed from, the mold and trimmed.

Thin sections of a light gold or silver reflection pattern (about 600 Å thick) were cut with a

glass knife. The sections were picked up on formvar-coated 200 mesh copper grids.

When required, the sections were stained with 2% aqueous uranyl acetate for 8 minutes, followed by Reynold's lead citrate (Reynolds, 1963) for 60 seconds. This staining method greatly increased the contrast of the specimen but was not desirable when the experimental conditions were employed. It was apparent that if the staining was intense, the experimental results tended to be obscured.

All grids were observed in the Philips 300 electron microscope at an accelerating voltage of 60 Kv. Negatives were taken at one-quarter second exposure time on Eastman Fine Grain Release Positive film and prints developed on Kodabromide paper of either 4 or 5 grade.

3. PREPARATION OF CONJUGATES

A. Wheat Germ Agglutinin-Horseradish Peroxidase (WGA-HRP)

This coupling procedure employed the one-step conjugation by glutaraldehyde (Avrameas, 1969) and followed the methods of Huet and Garrido (1972).

The lectin WGA (from Sigma or Miles Yeda) was used without any further purification. To a solution containing x mg of WGA, 2x mg HRP (Sigma Chemical Co.) were added in a fixed volume of 1 ml of 0.1 M phosphate buffer, pH 6.8. To cross-link these two molecules, glutaraldehyde was added to a final concentration of 0.03%. The reagents were gently swirled and allowed to react for 3 hours at room temperature.

Following the conjugation reaction, the WGA-HRP reaction mixture was placed in a small dialysis bag (prepared by boiling in d H_2O for 4 hours) and dialyzed against a large volume of buffer containing 2 mg/ml glycine overnight. Dialysis was performed to allow the excess glutaraldehyde to be removed from the reaction medium. The glycine was employed to inactivate the glutaraldehyde cross-linking within the reaction volume and also to inactivate the glutaraldehyde which would diffuse into the dialysate volume.

The dialysis was then continued with two changes of the buffer. This was the standard coupling reaction procedure, and when it was altered, the changes will be indicated.

The concentration of WGA-HRP applied to the embryos was found to be critical. Experiments tested 25 µg/ml, 50 µg/ml and 100 µg/ml of the conjugate. The results at 25 µg/ml were inconsistent, but at 50 µg/ml the results were repeatable, so 50 µg/ml of WGA-HRP was employed in all the experiments. In the controls, the conjugate was incubated for at least 2 hours with 0.5 M N-acetyl-glucosamine (GlcNAc, Sigma Chemical Co.) before being used.

The conjugate of WGA-HRP was localized on the cell surface by utilizing the technique of Graham and Karnovsky (1966) which makes the HRP electron dense by coupling it to 3'3' diaminobenzidine tetrahydrochloride (DAB). To a solution of 0.05 M Tris HCl buffer, pH 7.6, was added 2 mg/ml DAB (J.T. Baker). The solution was swirled with H_2O_2 (50% Fisher Scientific Co.) at a final concentration of 0.01%. The oxidation of the DAB was allowed to proceed for about 2 hours before it

was used as a stain. The activated DAB was allowed to react with the glutaraldehyde-fixed embryos for 30 minutes before post-fixation. The embryos were then rinsed three times for 10 minutes in the buffer and then post-fixed. The post-fixation with OsO_4 made the DAB deposit electron dense so that the molecules of WGA-HRP-DAB appeared as a dark deposit on the cell surface.

B. Wheat Germ Agglutinin-Ferritin (WGA-Fe)

The conjugation of this electron dense marker was a combination of two previous methods (Nicolson et al, 1975; Shimizu and Yamada, 1976). To a solution containing 5 mg ferritin (Sigma Chemical Co.) 2 mg WGA (Miles Yeda) and 11 mg GlcNAc in 1 ml 0.1 M phosphate buffer, pH 7.2, glutaraldehyde was added to a final concentration of 0.035% (W/V). The mixture was swirled and allowed to react for 3 hours at room temperature. Ethanolamine (Sigma Chemical Co.) pH 8, was added to a final concentration of 0.1 M and allowed to inhibit the crosslinking of glutaraldehyde for 30 minutes. The solution was then placed into a small dialysis bag (preboiled for 4 hours in d H_2O) and

dialyzed against 0.1 M phosphate buffer, pH 7.8 overnight.

Purification of the conjugate was performed by gel chromatography. The column preparation was as follows: 3 g CNBr-Sepharose (Pharmacia Fine Chemicals) was weighed out and washed, and swollen on a scintered glass filter with 600 ml of 1 mM HCl. Ovomucoid (Sigma Chemical Co.), 90 mg, was mixed with the gel in 0.1 M $NaHCO_3$, pH 8, plus 0.5 M NaCl for 2 hours at room temperature or overnight at 4°C. The excess ovomucoid was washed away with 0.1 M phosphate buffer, pH 7.2, and the remaining active groups of the CNBr-Sepharose were blocked with 1 M glycine, pH 9.0, for 2 hours at room temperature. The excess glycine was rinsed away with 0.1 M $NaHCO_3$, pH 8.3, plus 0.5 M NaCl, followed by acetate buffer (0.1 M, pH 4) and finally $NaHCO_3$ buffer. The gel was then stored at 4°C with sodium azide until use.

The ovomucoid-Sepharose was packed in a 0.9 x 15 cm column and equilibrated with 0.1 M KCl, 0.01 M Tris buffer, pH 7.4. The conjugate was absorbed to the column and then eluted with 0.05 M GlcNAc in d H_2O

or 0.1 M acetic acid, pH 2. The absorbance at 280 nm was monitored with a Beckman Model 26 spectrophotometer and recorder. The fraction containing the conjugate was collected and ultracentrifuged at 100,000 g for 2 hours. The precipitate pellet was then dissolved in a small volume of phosphate buffer and dialyzed overnight against phosphate buffer. Finally, to remove aggregates, the sample was fractionated on a small column of Sepharose 4B and the absorbance monitored at 280 nm. The first fraction which caused a peak at 280 nm was within the void volume of the column and was considered to be aggregates of the conjugate. The second peak was collected and utilized in the experiment. A Lowry protein determination was executed to calculate the final concentration of the conjugate.

4. NATIVE WGA VISUALIZATION

To study the distribution of native WGA, the technique of Francois et al (1972) was attempted. Native WGA, then purified lectin without any chemical modifications (100 µg/ml) in the buffer, was incubated with the embryos for 1 hour. In the control

situation, 100 µg/ml WGA plus 0.5 M GlcNAc was incubated with the glutaraldehyde-fixed embryos for 1 hour at room temperature. This incubation was followed by two rinses with the buffer for 15 minutes each. The embryos were then incubated with 1 mg/ml HRP for 1 hour at room temperature. Then they were rinsed four times with the buffer for 15 minutes each and allowed to react with DAB for 30 minutes at room temperature. Finally, the embryos were rinsed three times for 10 minutes each with the buffer and post-fixed.

This experiment was to test the distribution of WGA on the surface of the embryo. The WGA was not modified by conjugation in this case, so that it could be called native WGA binding. The rationale was that if the conjugation reaction was altering the binding site of the WGA, the distribution of the native WGA binding would be different than the conjugated WGA.

In addition, native WGA was visualized on the cell surface with the method of Francois and Mongiat (1977). WGA (50 µg/ml) in the buffer was incubated with the embryos for 30 minutes at room temperature.

As a control, WGA plus 0.5 M GlcNAc was incubated with the embryos for 30 minutes at room temperature. This was followed by three rinses for 10 minutes with the buffer. Then the embryos were incubated with 1 mg/ml glucose oxidase (Sigma Chemical Co.) for 15 minutes at room temperature, followed by two rinses for 10 minutes with the buffer. The glucose oxidase was revealed histochemically via the method of Kuhlmann and Avrameas (1971), which uses 15 mg glucose, 0.5 mg DAB and 1 mg HRP per ml of 0.1 M phosphate buffer, pH 6.8. This reaction mixture was incubated with the embryos following glutaraldehyde fixation and three thorough rinses (20 minutes each) with the buffer. The incubation was at 37°C and lasted for at least 30 minutes (for increased reaction product, the incubation was allowed to proceed up to 1 hour). The resulting deposit was electron dense.

5. ENZYME PRETREATMENT OF THE CELL SURFACE (STAGE 1 EMBRYOS)

The cell surface was pretreated with neuraminidase (Nanase, Schwartz-Mann) prior to the

binding of the WGAHRP conjugate. This enzyme, from Vibrio cholerae, has optimum activity at pH 5.7. It requires the presence of Ca++ for its hydrolysis activity at α-2,6 links between the terminal N-acetylneuraminic acid and 2 acetamido-2-deoxy-D-galactose. However, with prolonged incubation, this enzyme will cleave all the sialic acid from terminal positions on a variety of substrates without preference for linkage type. The embryos were incubated in PCS containing 20 units/ml Nanase for up to 1 hour at 37°C. Then they were rinsed with cold buffer twice for 10 minutes each and fixed with 2.5% glutaraldehyde.

The cell surface was also pretreated with crude bovine testis hyaluronidase (HAse, Sigma Chemical Co., Grade III). This enzyme preparation is very crude and contains other enzymes as well as HAse. However, it will degrade hyaluronic acid, chondroitin and the chondroitin sulfates with a pH optimum of 5.0. The embryos were pretreated with crude HAse (50 µg/ml or 22.5 units/ml) for 30 minutes at pH 5.0 and 37°C. The embryos were then rinsed and fixed with 2.5% glutaraldehyde.

To study the amount of WGA-HRP binding to hyaluronic acid (HA) only, HAse from Streptomyces (Calbiochem) was used to eliminate this glycosaminoglycan (GAG). This enzyme degrades only HA and liberates GlcNAc and glucuronic acid with breakdown products of these two carbohydrates (Ohya and Kaneko, 1970). It has a pH optimum of 5.0 and an optimum temperature of 60°C. Approximately 12.5 or 16.6 TRU/ml (turbidity reducing units) of HAse in 0.01 M sodium acetate, pH 5.0, plus 0.1 M NaCl was incubated with the unfixed embryo for 60 minutes at 37°C. As a control experiment, the unfixed embryo was incubated in the same medium but without the HAse for 60 minutes at 37°C. Following the incubation, the embryos were gently rinsed two times for 10 minutes each with cold buffer and fixed with 2.5% glutaraldehyde. In those experiments that employed pretreatment with HAse, the embryos were rinsed once before incubation with a buffer solution that was identical to the vehicle carrying the enzyme.

In addition, the effect of trypsin and pronase upon the binding of WGA-HRP to chick embryos was studied. These two enzymes act only upon the proteins

extending from the cell surface. Pronase has a broad range of activity and cleaves proteins randomly. On the other hand, trypsin will cleave only at lysine and arginine amino acids. However, both of these enzymes should decrease the amount of carbohydrate upon the cell surface. Specifically, 0.1% trypsin (1:300, Becton, Dickinson & Co.) in 0.1 M phosphate buffer, pH 7.4, was incubated with the embryos for 15 or 30 minutes at 37°C. Other embryos were incubated with 0.1% pronase (Calbiochem, B grade) in 0.1 M phosphate buffer, pH 7.4, for 15 or 30 minutes at 37°C. The embryos were then rinsed briefly with phosphate buffer and fixed with 2.5% glutaraldehyde.

In the control experiments, there was no enzyme in the medium in which the embryos were incubated. However, the medium containing the embryo was the same as the vehicle carrying the enzyme. following these pretreatments, Stage 1 embryos were allowed to react with 50 µg/ml of the WGA-HRP conjugate (0.03% glutaraldehyde) for 30 minutes at room temperature. The embryos were then rinsed with buffer,

post-fixed and prepared for observation in the electron microscope.

RESULTS

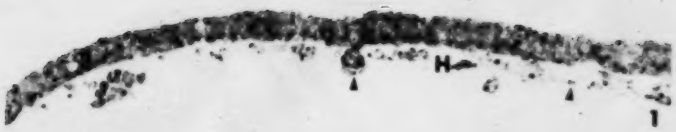
The general morphology of the chick embryo when it leaves the uterus of the hen has been examined and staged by Eyal-Giladi and Kochav (1976). They suggest that when the egg is expelled, the actual development, and hence morphology, of the embryo will differ from one individual to another. It appears that the major variable within these few critical hours is the relative development of the hypoblast. The epiblast appears throughout these early stages as a columnar sheet of epithelial-like cells that are about 9-12 μ in diameter and have tight apical junctions (Figure 5). A Stage X embryo has isolated groups of hypoblast cells that may be about 10-19 μ in diameter, contain large amounts of yolk granules and have a round morphology without any apparent junctional complexes (Figure 1). However, once the "sickle of Koeller", or crescent of hypoblast cells, forms at the posterior end of the embryo, the hypoblast cells have altered their morphology. The Stage XII embryo has hypoblast cells that are thin and extended, like a squamous layer of cells (Figure 6). Their relative amount of

Fig. 1 Light micrograph of a cross-section through the center of a Stage 1 chick embryo (Hamburger & Hamilton, 1951) stained with Toludine blue (x 400). E-epiblast cells, H-hypoblast cells. Note within the hypoblast there are two types of cells: large round cells and flat extended cells (indicated with arrows).

Fig. 2 Light micrograph of a cross-section through the primitive streak of a Stage 5 chick embryo (Hamburger & Hamilton, 1951) stained with Toludine blue (x300). E - epiblast cells, M - mesenchyme cells, N - endoblast cells, PS - primitive streak.

Fig. 3 Higher magnification of a cross-section through the primitive streak stained with Toludine blue (x750). E - epiblast cells, M - mesenchyme cells, N - endoblast cells, PS - primitive streak.

Fig. 4 Higher magnification of a cross-section through an area lateral to the primitive streak stained with Toludine blue (x750). E - epiblast cells, M - mesenchyme cells, N - Endoblast cells.



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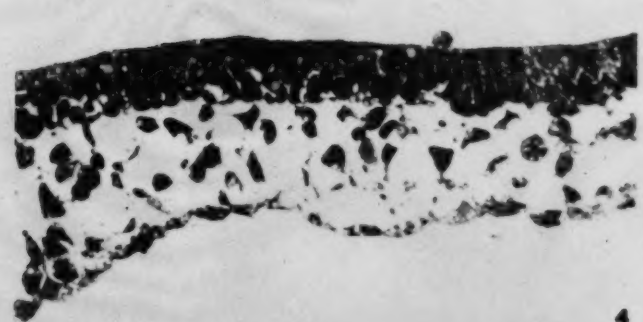

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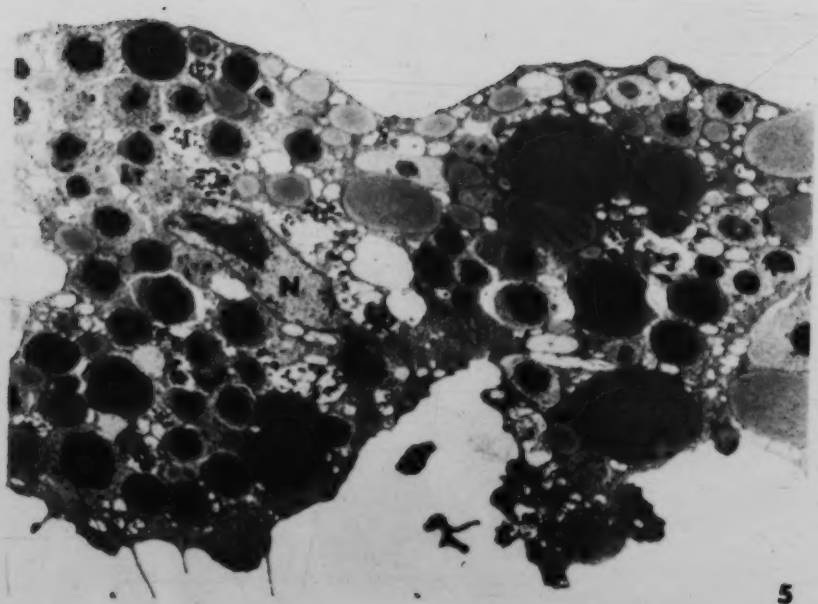
Fig. 5 Electron micrograph of the epiblast cells in a Stage 1 chick embryo (Hamburger & Hamilton, 1951) stained with uranyl acetate and lead citrate (x7,600). N - nucleus.

Fig. 6 Electron micrograph of a cross-section through the hypoblast cells in the "sickle of Koeller" of the Stage 1 embryo (Hamburger & Hamilton, 1951). Stained with uranyl acetate and lead citrate (x7,600).

Note: the cells have a flattened morphology.



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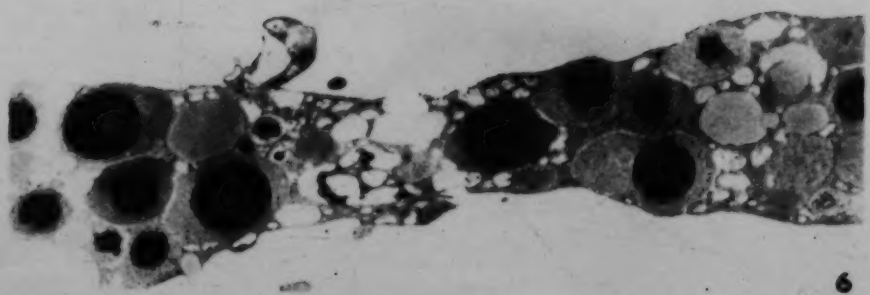

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yolk granules appears to have diminished and they seem to have formed more specialized cellular contacts between adjacent cells.

Although there appeared to be morphological differences between individual unincubated embryos, for the purpose of cell surface changes associated with development, all of the unincubated embryos will be classed as Stage 1 (Hamburger & Hamilton, 1951). The classification into Stage 1 is possible because none of the results showed any differences between these early stages (according to Eyal-Giladi and Kochav) with regard to the cell surface. However, after the embryo has developed to Stage 4 or 5 (Hamburger & Hamilton, 1951), the cell surface and the morphology have changed (Figures 2, 3, and 4).

Throughout the experiments, the embryos were sectioned in two areas. The center of the area pellucida of Stage 1 and Stage 5 embryos was examined first. Then the lateral areas of the area pellucida were examined. It was assumed that cells from these two areas would show the greatest variance in surface structure as reflected by WGA binding. The results

indicate that the Stage 1 embryo had no difference in the surface distribution of WGA binding sites from these two areas. Therefore, representative micrographs are only from the central region. However, once the embryo had progressed to Stage 5, there was an apparent difference in the surface distribution of the WGA binding sites according to the region examined.

A variety of methods was employed to localize the WGA binding sites upon the cell surfaces. The two most successful methods of tagging the WGA were the direct method of a one-step conjugation of WGA to HRP using glutaraldehyde, and the indirect method of binding glucose oxidase to WGA previously bound to the cell surface. When the WGA and its marker are reacted with the tissue, together or as a unit, this is called the "direct method". However, when the WGA is allowed to react with the tissue and after a rinse period the marker is allowed to react with the WGA that has remained on the tissue, this is called the "indirect method". Both procedures should produce the same result, with the presence of WGA being revealed upon the cell surface of the tissue by the activity of the marker enzyme. With few exceptions, all controls were

carried out using N-acetyl-glucosamine (GlcNAc) as the hapten inhibitor. Where no binding of WGA was seen in these controls, it was assumed that the cell surfaces binding WGA in experimental samples possessed exposed GlcNAc residues. In several cases, the hapten inhibitor failed to prevent WGA binding in the controls. In these latter cases, the specificity of the experimental binding was questioned and examined further.

1. Unincubated Chick Embryos (Stage 1)

In these experiments, the embryos were either prefixed with 2.5% glutaraldehyde for 2 hours at 4°C and then manipulated, or they were manipulated as live tissue and then fixed following the same procedure. Neither procedure seemed to alter the distribution of the WGA binding sites in general, although the prefixation retained the structure of the embryo *in Situ*.

A. WGA-HRP conjugated with 0.03% glutaraldehyde (fixed and unfixed tissue)

The distribution of WGA binding sites is reflected by an electron dense deposit located along the extracellular side of the plasma membrane. This electron dense deposit is the reaction product formed by the deposition of diaminobenzidine wherever the HRP occurs. Naturally, since the WGA is conjugated to the HRP, the deposit reflects the WGA binding sites. In the control experiment, the WGA-HRP conjugate is allowed to react with its appropriate hapten inhibitor, GlcNAc. Although the conjugate and the hapten reacted for at least 2 hours, there was a consistent, residual, non-specific binding to the dorsal surface of the epiblast (DE Fig. 7) and the ventral surface of the hypoblast (VH Fig. 11). The other surfaces had their reaction product removed by the hapten in the control experiments (Figs. 8, 9 and 10). This indicates that the WGA binding sites on the lateral surfaces of the epiblast (LE), ventral surfaces of the epiblast (VE), and dorsal surfaces of the hypoblast (DH), are specifically due to the presence of GlcNAc (Figs. 13, 14 and 15 respectively). Due to the WGA binding in the controls, little can be deduced from the binding by the DE (Fig. 12) and VH (Fig. 16) in these experiments.

Fig. 7 to 11 Electron micrographs of the cell surfaces of the prefixed Stage 1 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.03% glutaraldehyde) + 0.5 M GlcNAc for 30 minutes at room temperature and then rinsed.

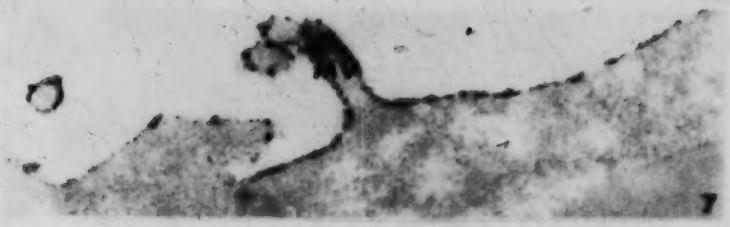
Fig. 7 The dorsal surface of the epiblast, unstained (x 37,100). MV-microvilli.

Fig. 8 The lateral surfaces of the epiblast, unstained (x 37,100). M-mitochondrion.

Fig. 9 The ventral surface of the epiblast, unstained (x 37,100).

Fig. 10 The dorsal surface of the hypoblast, unstained (x 37,100).

Fig. 11 The ventral surface of the hypoblast, unstained (x 37,100). M-mitochondrion.



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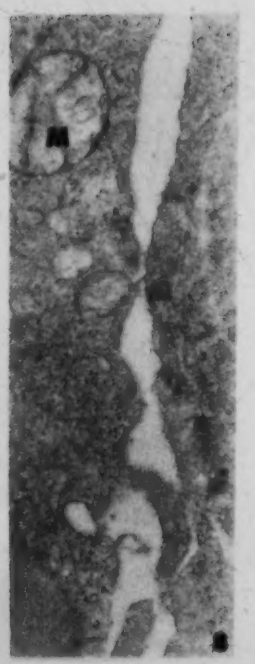

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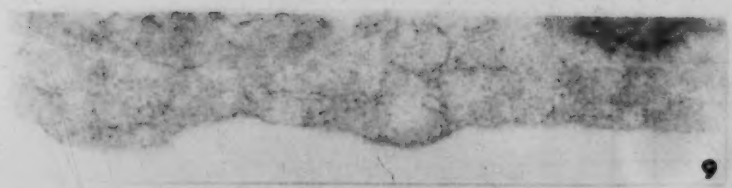

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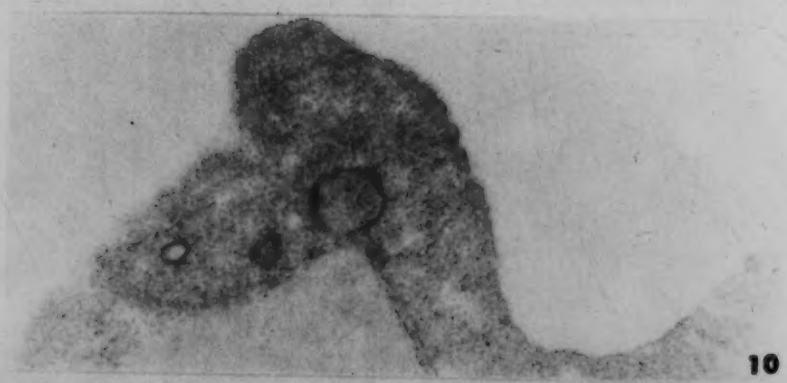

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Figs. 12 to 16 Electron micrographs of the cell surfaces of the prefixed Stage 1 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.03% glutaraldehyde) for 30 minutes at room temperature and then rinsed.

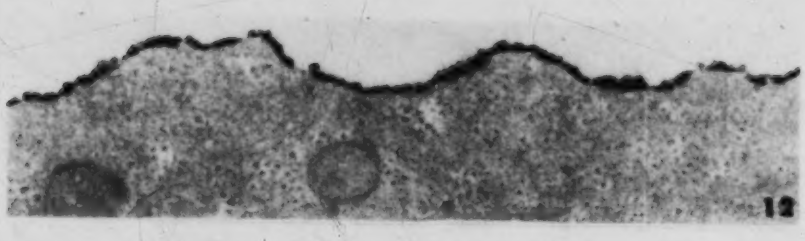
Fig. 12 The dorsal surface of the epiblast, unstained (x 37,100).

Fig. 13 The lateral surfaces of the epiblast, unstained (x 37,100). Arrows indicate WGA binding sites.

Fig. 14 The ventral surface of the epiblast, unstained (x 37,100). ER-endoplasmic reticulum; arrows indicate WGA binding sites.

Fig. 15 The dorsal surface of the hypoblast, unstained (x 37,100). Arrows indicate WGA binding sites.

Fig. 16 The ventral surface of the hypoblast, unstained (x 37,100).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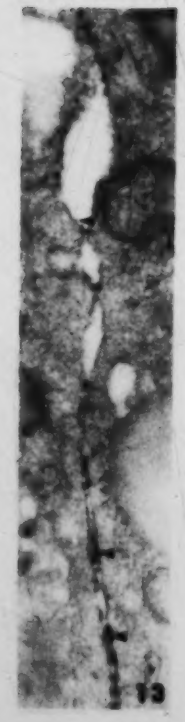
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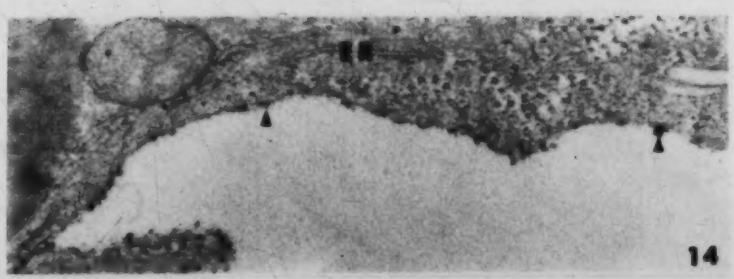
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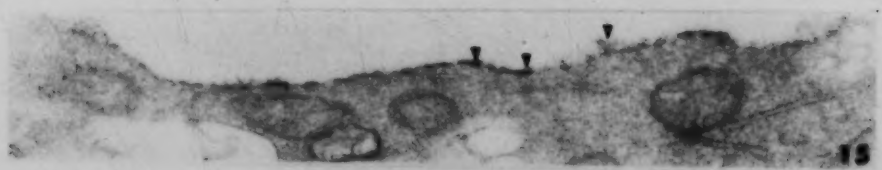
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The failure of the control experiments with regard to DE and VH prompted further study to explain the non-specificity of the WGA-HRP conjugate. The following procedures were adopted:

(i) Altering the final concentration: The final concentration of the conjugate was adjusted between 25 µg/ml and 100 µg/ml. At the lowest concentration, the binding pattern of WGA was erratic and the controls inconsistent. When the final concentration was 100 µg/ml, there was an associated increase in the non-specific binding to the DE and VH (Figs. 17 to 20). Over a series of experiments, the desired consistency of results in the experimental situation and the least non-specific binding occurred when the final concentration of the WGA-HRP conjugate was 50 µg/ml.

(ii) Activated HRP: Whether the non-specificity of the conjugate was due to the HRP moiety was tested by reacting the HRP with glutaraldehyde in the absence of WGA, then dialyzing and reacting the activated HRP with the chick embryo. There was no binding of HRP to the surfaces non-specifically. In

Figs. 17 to 20 Electron micrographs of the cell surfaces of the unfixed Stage 1 chick embryo treated with 100 µg/ml WGA-HRP (conjugated with 0.03% glutaraldehyde) for 30 minutes at room temperature.

Fig. 17 The dorsal surface of the epiblast, experimental, unstained, (x 37,100).

Fig. 18 The dorsal surface of the epiblast, control experiment (treatment was with 100 ug/ml WGA-HRP preincubated with 0.5 M GlcNAc), unstained (x 37,100).

Fig. 19 The ventral surface of the hypoblast, experimental, unstained (x 37,100).

Fig. 20 The ventral surface of the hypoblast, control experiment, unstained (x 37,100).

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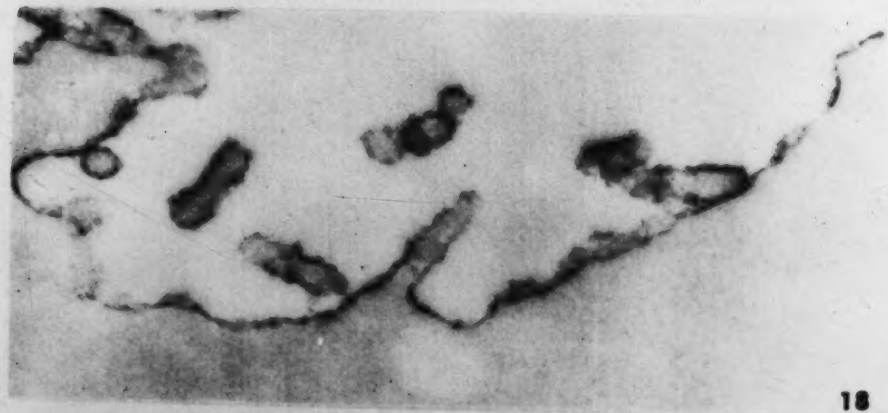

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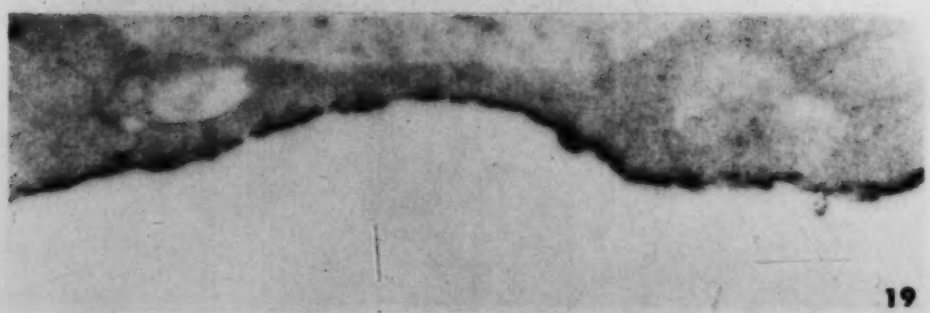

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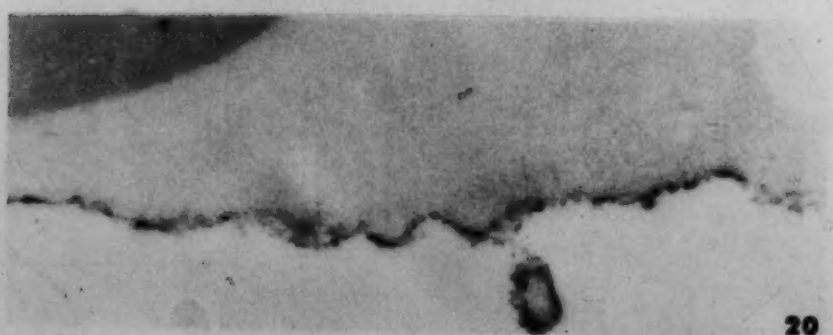

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addition, due to the absence of reaction product, it may be inferred that the subsequent intensification reactions with diaminobenzidine do not stain the cell surfaces non-specifically. These last two findings suggested that the non-specific adsorption of the conjugate was due to the lectin, WGA, and not the enzyme marker systems.

(iii) Hapten concentration and combinations: Another possible explanation for the non-specific binding in the controls was that the relatively high concentrations of the hapten (Ø.5 M) were inducing the two surfaces (DE and VH) to absorb the conjugate non-specifically, by altering the surface charge or by a sugar transporting mechanism at these sites. The high concentration of the hapten may also induce the WGA to increase any non-specific adsorption. Also, GlcNAc by itself may not be a potent enough inhibitor, but with the presence of another hapten, the two may inhibit binding. The other sugar moiety that was chosen was N-acetyl-galactosamine (GalNAc) due to its similarity of structure. Experiments were conducted with the following combinations: Ø.5 M

GlcNAc; 0.1 M GlcNAc; 0.5 M GlcNAc + 0.5 M GalNAc; 0.1 M GlcNAc + 0.1 M GalNAc. All results appeared identical to the control experiment with the hapten GlcNAc at a concentration of 0.5 M (Figs. 7 to 16).

(iv) Glycine rinse: The problem with the glutaraldehyde fixation is that unreacted free aldehyde groups within the membrane remain active for some time following fixation. To eliminate possible non-specific reactivity of glutaraldehyde with WGA, the tissue was washed after fixation with phosphate buffer containing 2 mg/ml glycine twice, and then rinsed with buffer alone. Although the active aldehyde groups of the glutaraldehyde would have been eliminated by the reaction with the glycine during washing, the experiments had identical results to the initial attempts (Figs 7 to 16).

(v) WGA source: The commercial product WGA was tested and compared between two companies. There was evidence that a variety of purification procedures had been employed so that the end product could conceivably have an altered specificity. There-

fore, WGA was purchased from Sigma to compare with the results of the previous binding studies with the WGA from Miles. Both lectins were used similarly and conjugated to HRP with 0.03% glutaraldehyde. However, there appeared to be no difference in the binding patterns of one lectin compared to the other. Essentially the results were similar to previous findings (Figs. 7 to 16).

(vi) Live tissue: The effect of WGA binding and the distribution of WGA binding sites upon live tissues were examined. In this experiment, the WGA (Sigma)-HRP conjugate was allowed to react with live Stage 1 embryos for 15 minutes at room temperature before a 60 minute rinsing period and subsequent fixation with glutaraldehyde. The results indicated that the non-specific binding to the DE and VH was still present (Figs. 21 to 25). Also, there appeared to be some non-specific binding to the VE (Fig. 22). The cellular response to the non-specific binding is the same as the response to the binding without the hapten present (Figs. 26 to 30). The reaction product can be visualized as being clumped or patched and

Figs. 21 to 25 Electron micrographs of the cell surfaces of the unfixed Stage 1 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.03% glutaraldehyde) + 0.5 M GlcNAc for 15 minutes at room temperature, rinsed three times at 4° C and then fixed.

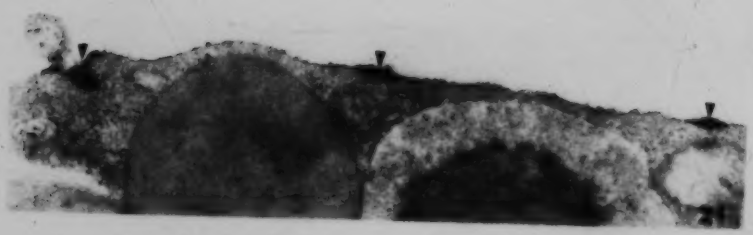
Fig. 21 The dorsal surface of the epiblast, unstained (x 37,100). Arrows indicate patching of the non-specific WGA binding sites.

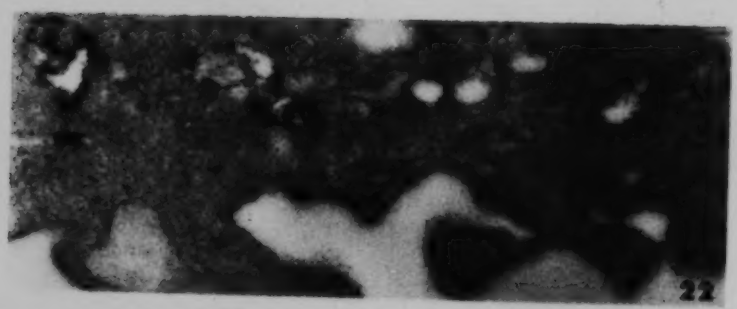
Fig. 22 The ventral surface of the epiblast, unstained (x 37,100). EV-endocytotic vesicles containing non-specific WGA binding sites.

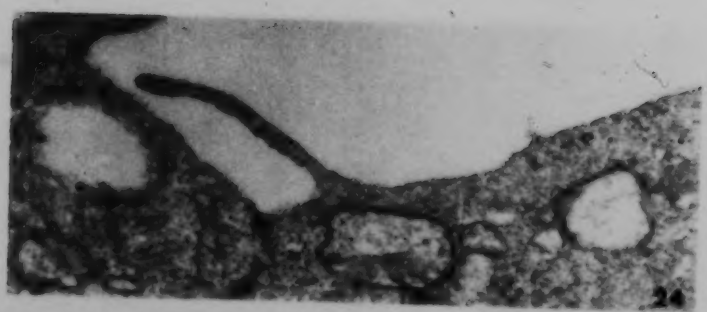
Fig. 23 The lateral surface of the epiblast, unstained (x 37,100).

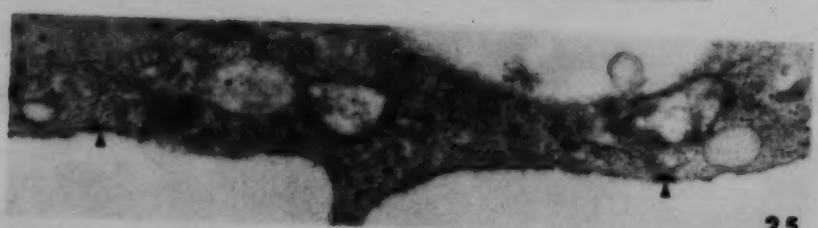
Fig. 24 The dorsal surface of the hypoblast, unstained (x 37,100).

Fig. 25 The ventral surface of the hypoblast, unstained (x 37,100). Arrows indicate non-specific WGA binding sites.









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is seen within vacuoles in the cytoplasm; apparently the WGA-HRP has been endocytosed and removed from the cell surface (DE Fig. 26). In subsequent experiments, the time of exposure of the tissue to the WGA conjugate was increased and the rinsing time decreased. Here the redistribution of the binding was enhanced and more surfaces had active endocytosis (i.e. DE, VE and VH).

(vii) Protecting the active site: In this set of experiments, WGA (Miles) was allowed to react with 0.05 M GlcNAc before reacting with HRP and 0.03% glutaraldehyde. This modification was incorporated to see if the HRP was binding to the active site of the WGA molecule and disturbing its binding patterns. The hapten was subsequently removed from the lectin conjugate by extensive dialysis against large volumes of low ionic strength buffer. The protection of the binding site had no effect upon the relative distribution of WGA binding sites, as revealed with this conjugate. The results of the binding of the site-protected conjugate are essentially the same as those in Figs. 7 to 16.

Figs. 26 to 30 Electron micrographs of the cell surfaces of the unfixed Stage 1 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.03% glutaraldehyde) for 15 minutes at room temperature, rinsed three times at 4° C and then fixed.

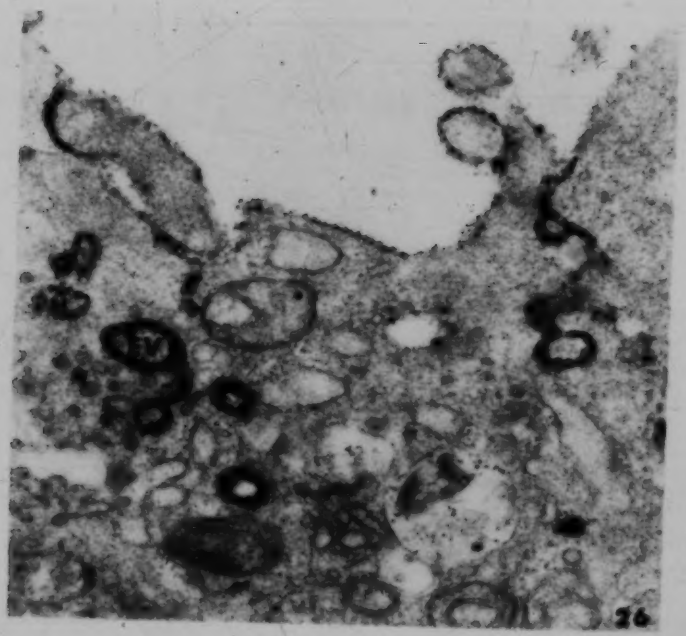
Fig. 26 The dorsal surface of the epiblast, unstained (x 37,100) EV-endocytotic vesicles.

Fig. 27 The ventral surface of the epiblast, unstained (x 37,100). Arrows indicate WGA binding sites.

Fig. 28 The lateral surface of the epiblast, unstained (x 37,100).

Fig. 29 The dorsal surface of the hypoblast, unstained (x 37,100). Arrows indicate WGA binding sites.

Fig. 30 The ventral surface of the hypoblast, unstained (x 37,100). Arrows: patching or clumping of the WGA binding sites.



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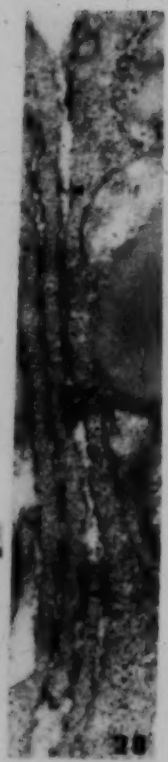

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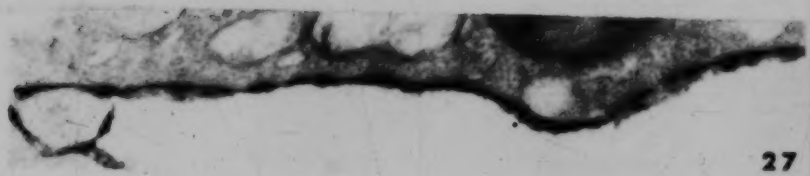

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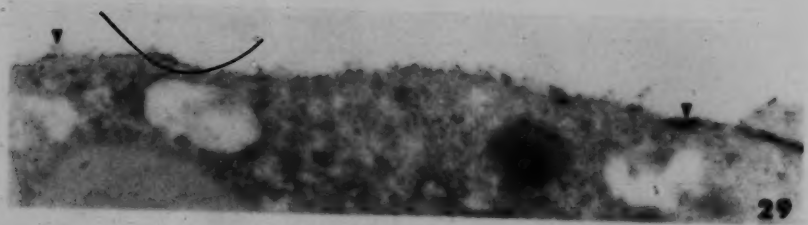

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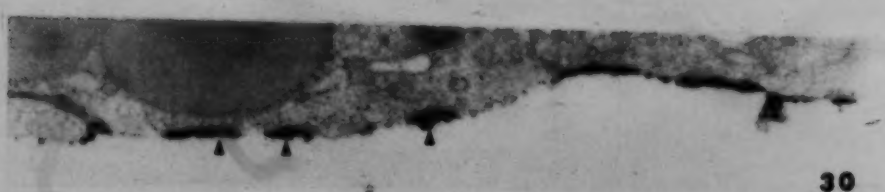

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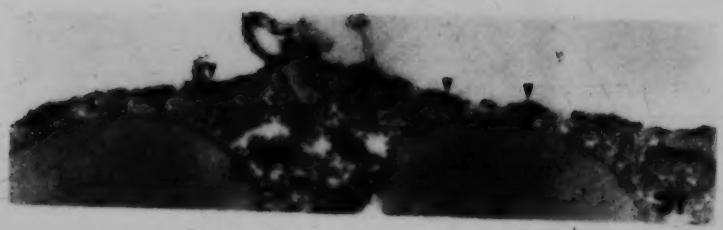
(viii) Temperature: Experiments were conducted to examine the effect of binding at different temperatures upon live tissue. Essentially, the relative amounts of specific and non-specific binding remained constant. However, the major effect of temperature was upon the distribution of the binding sites. When the embryos were incubated for 30 minutes at room temperature in the presence of WGA-HRP, the electron dense deposit was actively endocytozed by the DE. However, when the binding occurred at 4°C the endocytosis had been reduced but the patching response remained. An even more dramatic effect of temperature upon the redistribution of WGA binding sites in live tisue was obtained by incubating embryos at 37°C for 10 minutes in the presence of the WGA-HRP conjugate or the conjugate and its hapten. The controls had a similar non-specific distribution of deposit upon the DE and VH, with slight clumping upon both surfaces. The experimentals (Figs. 31 to 33) had greater uptake of conjugate by the VE than the controls (Fig. 33). The results for the temperature effect upon redistribution consistently produced results that were comparable to the

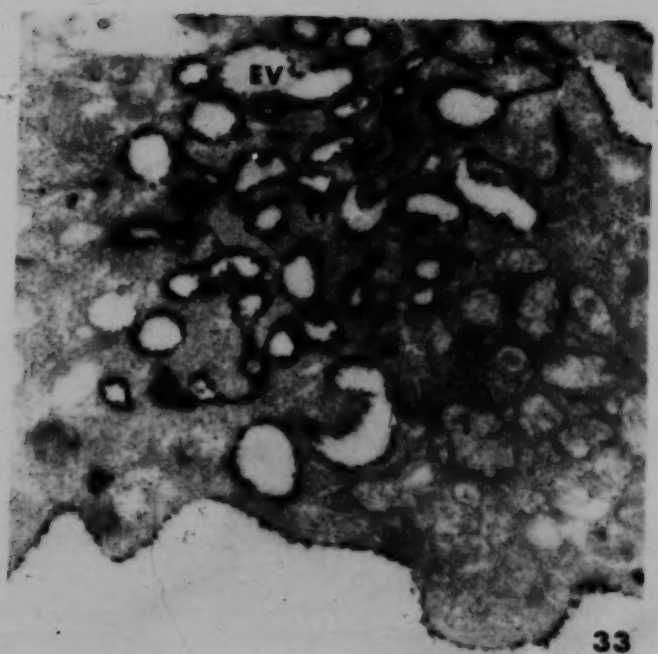
Figs. 31 to 33 Electron micrographs of the cell surfaces of the unfixed Stage 1 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.03% glutaraldehyde) for 10 minutes at 37_o C, rinsed three times at 4_o C and then fixed.

Fig. 31 The dorsal surface of the epiblast, unstained (x 37,100). Arrows show patching or clumping of the WGA binding sites.

Fig. 32 The lateral surface of the epiblast, unstained (x 37,100).

Fig. 33 The ventral surface of the epiblast, unstained (x 37,100). EV-endocytotic vesicles.





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initial experiments with the WGA-HRP conjugate, i.e. non-specific binding to the DE and VH. (Figs. 7 to 16).

(ix) Enzyme pretreatment: The apparent non-specific binding in the control experiments was consistent in the sense that it occurred always on the DE and VH. These results prompted further investigation of the nature of the binding of the WGA-HRP conjugate to DE and VH. Live embryos were pre-incubated with a series of enzymes:

(a) neuraminidase (V. cholera), 20 units/ml for 30 to 60 minutes at 37°C;

(b) testicular hyaluronidase type III, 22.5 units/ml for 30 minutes, pH 5, at 37°C;

(c) testicular hyaluronidase type VI, 40 units/ml for 30 minutes, pH 5, at 37°C;

(d) hyaluronidase, Fungal, 50 µg/ml for 30 to 60 minutes, pH 5, at 37°C;

(e) hyaluronidase, Fungal, 50 µg/ml for 120 minutes, pH 7.4 at 37°C;

(f) trypsin, 0.1% (1 mg/ml), 15 and 30 minutes, pH 7.4, at 37°C;

(g) pronase, 0.1% (1 mg/ml), 15 and 30 minutes, pH 7.4, at 37°C.

The results are listed in Table 1. It was found that only crude testicular hyaluronidase removed the non-specific binding. All the other enzymes seemed to have very little, if any, effect upon the non-specific binding of the WGA-HRP conjugate. Neuraminidase was used because of the known non-specific binding of sialic acid by WGA (Greenaway and LeVine, 1973); hyaluronidase was used because of the presence of GlcNAc within the carbohydrate portions of the glycosaminoglycan, hyaluronic acid; and trypsin and pronase were used to remove large portions of the surface proteins and glycoproteins. Neuraminidase, trypsin and pronase decreased the amount of binding of WGA-HRP to all surfaces, but all failed to remove the non-specific binding sites. The latter two enzymes had a greater effect when they were allowed to react with the tissue for 30

TABLE 1. EFFECT OF ENZYME PRETREATMENT UPON WGA-HRP BINDING

Enzyme	Treatment (units/ml) at 37°C	WGA-HRP + 0.5 M GlcNAc on the		WGA-HRP on the	
		DE	VH	DE	VH
Control	none	+ → ++	+ → ++	++	++
Nanase	20 at pH 7.4 for 30 minutes	+	+	++	++
	for 60 minutes	+	+	++	++
HAse (III)	22.5 at pH 5.0 for 30 minutes	0	0	+	+
HAse (VI)	40 at pH 5.0 for 30 minutes	+	+	++	++
HAse fungal (*Streptomyces*)	50 µg/ml at pH 5.0 for 30 minutes	+	+	++	++
	for 60 minutes	+	+	++	++
HAse fungal (*Streptomyces*)	50 µg/ml at pH 7.4 for 120 minutes	+ → ++	+ → ++	++	++
Trypsin	1% at pH 7.4 for 15 minutes	+ → ++	+ → ++	++	++
	for 30 minutes	not done	not done	+	+
Pronase	1% at pH 7.4 for 15 minutes	+ → ++	+ → ++	++	++
	for 30 minutes	not done	not done	+	+

Explanation of the symbols: 0 = no electron dense deposit, e.g. FIG. 9
+ = light amount of deposit, e.g. FIG. 11
++ = heavy amount of deposit, e.g. FIG. 12

Table 1

minutes. Interestingly, broad spectrum hyaluronidase effectively removed the non-specific binding, while enzymes with greater specificity for hyaluronidate residues failed to produce any effect. The incubation at 37°C in a medium of pH 5 drastically affected the embryos, but the membranes were intact and WGA-HRP was visualized on the surface in the experimental situation of incubation at pH 5 without any enzyme treatment. The failure of the enzyme pretreatment suggested that the binding of the WGA-HRP in the control experiments was non-specific. However, only the DE and VE had the non-specific attraction for the conjugate.

B. WGA-HRP conjugated with 0.01% glutaraldehyde (fixed tissue)

To limit the effects of the cross-linking of glutaraldehyde upon the specificity of the WGA molecule, the amount of glutaraldehyde used during the conjugation procedure was decreased to a final concentration of 0.01%. The remaining procedures were the same as in the previous experiments. The non-specific binding in the controls did not occur with this conjugate. When 0.5 M GlcNAc was used as the hapten inhibitor in the controls, there was no

apparent reaction product on any surface of the unincubated chick embryo (Figs. 34 to 38), i.e. all the binding in the experimental procedures was considered specific for terminal GlcNAc residues.

Therefore, as reflected by the distribution of the electron dense reaction products on Figs. 39 to 43, all surfaces of the unincubated chick embryo possessed WGA binding sites that are specific for terminal GlcNAc residues.

It was concluded that because of the apparent specificity of the WGA-HRP conjugated with 0.01% glutaraldehyde, the non-specific binding consistently seen in the previous experiments was due to alteration of the WGA molecule by 0.03% glutaraldehyde in the coupling reaction. The non-specific binding seen earlier may have been either from non-specific adsorption of the conjugate to the DE and VH or from binding of the conjugate to a site that has a higher affinity for the 0.03% conjugated lectin than the hapten (GlcNAc).

Figs. 34 to 38 Electron micrographs of the cell surfaces of the prefixed Stage 1 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.01% glutaraldehyde) + 0.5 M GlcNAc for 30 minutes at room temperature and then rinsed.

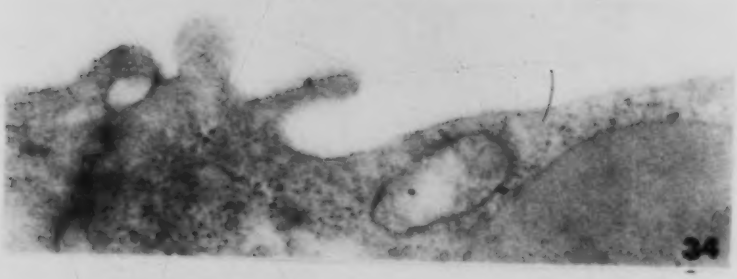
Fig. 34 The dorsal surface of the epiblast, unstained (x 37,100).

Fig. 35 The lateral surface of the epiblast, unstained (x 37,100).

Fig. 36 The ventral surface of the epiblast, unstained (x 37,100).

Fig. 37 The dorsal surface of the hypoblast, unstained (x 37,100).

Fig. 38 The ventral surface of the hypoblast, unstained (x 37,100).



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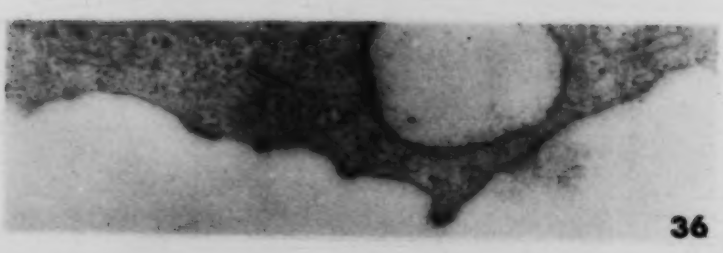

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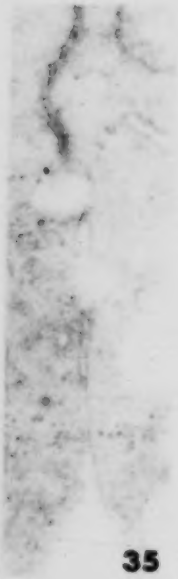

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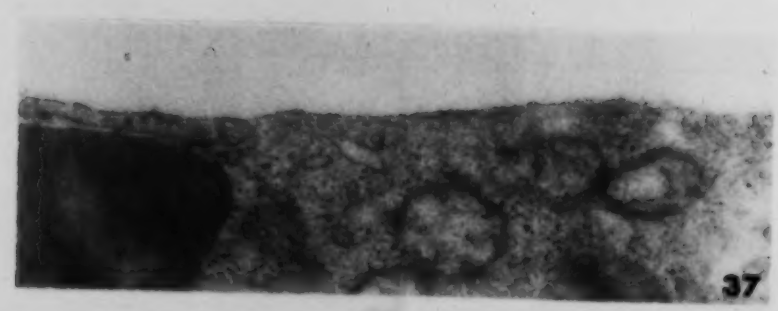

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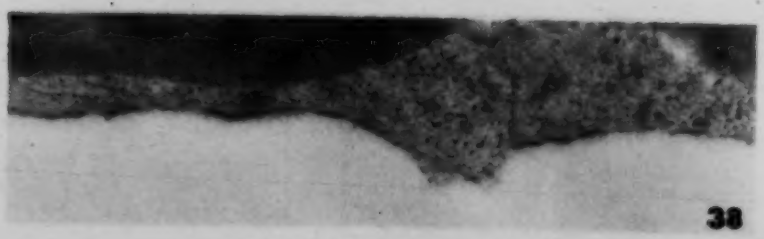

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Figs. 39 to 43 Electron micrographs of the cell surfaces of prefixed Stage 1 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.01% glutaraldehyde) for 30 minutes at room temperature and then rinsed.

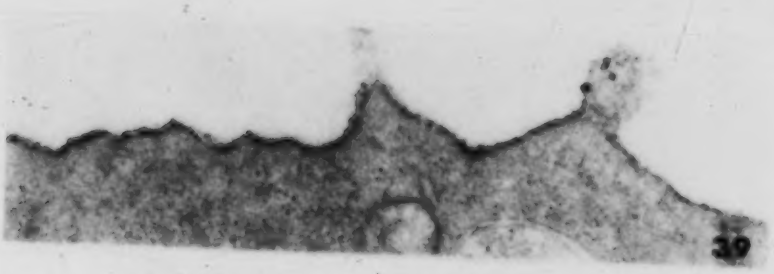
Fig. 39 The dorsal surface of the epiblast, unstained (x 37,100)

Fig. 40 The lateral surface of the epiblast, unstained (x 37,100).

Fig. 41 The ventral surface of the epiblast, unstained (x 37,100).

Fig. 42 The dorsal surface of the hypoblast, unstained (x 37,100).

Fig. 43 The ventral surface of the hypoblast, unstained (x 37,100).



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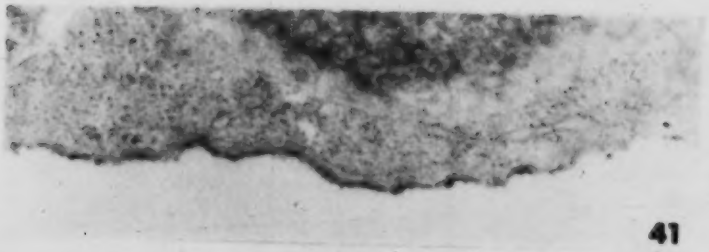

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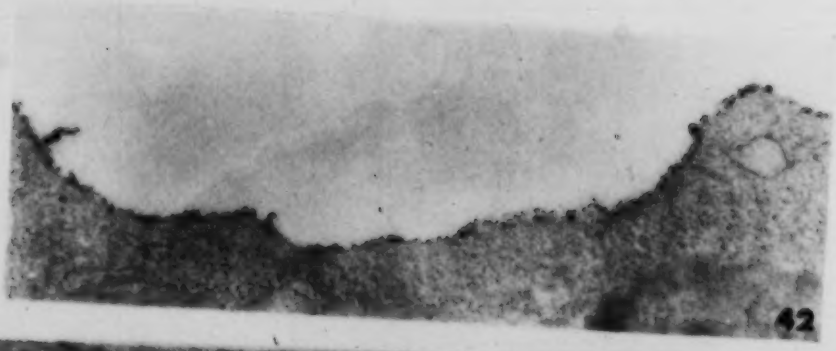

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C. WGA conjugated to ferritin (fixed tissue)

The preparation of WGA conjugated to ferritin resulted in a great loss of lectin conjugate. The concentration during the initial conjugation reaction was 2 mg/ml. The concentration after the final purification reaction was 25 µg/ml (as estimated by the Lowry protein determination method). The results of the reaction of 25 µg/ml WGA-Fe with the tissue are inconsistent (Figs. 44 and 45). Although there appeared to be binding to only the DE and VH, the concentration of the WGA-Fe was so low that conclusions are difficult to draw. Furthermore, the failure of the hapten to remove the binding from the DE and VH would again suggest that there is non-specific binding resulting from the conjugation procedure. In conclusion this technique needed an improvement during the preparation of the WGA-Fe conjugate in order to increase the yield, so that the distribution of binding sites could be analyzed.

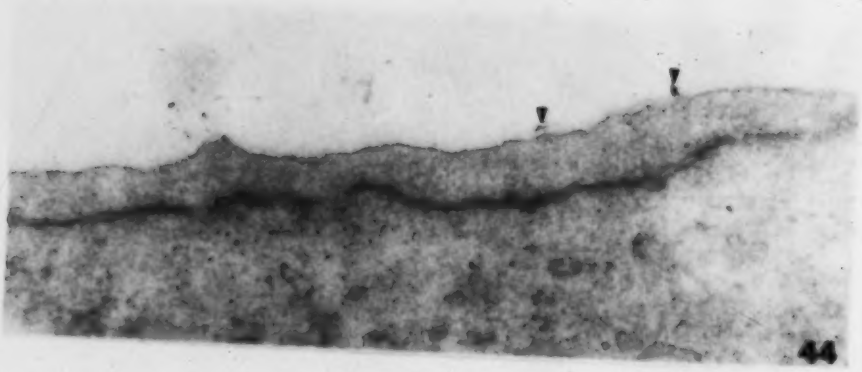
D. WGA localized by incubation with HRP (unfixed tissue)

According to Francois et al (1972), WGA is localizable at the cell surface by the indirect labelling of

Fig. 44 The dorsal surface of the epiblast of the prefixed Stage 1 chick embryo treated with approximately 25 µg/ml WGA-Fe for 30 minutes at room temperature, unstained, (x 55,650). Arrows indicate Fe marker.

Fig. 45 The ventral surface of the epiblast of the prefixed Stage 1 chick embryo treated with approximately 25 µg/ml WGA-Fe for 30 minutes at room temperature, unstained (x 55,650). Arrows indicate Fe marker.

Fig. 46 Electron micrograph of the ventral cell surface of the epiblast treated with 50 ug/ml WGA + 0.5 M GlcNAc, rinsed twice, then incubated with 1 mg/ml HRP (to reveal the WGA on the cell surface) and finally rinsed and fixed. Unstained (x 37,100). EV-endocytotic vesicles.



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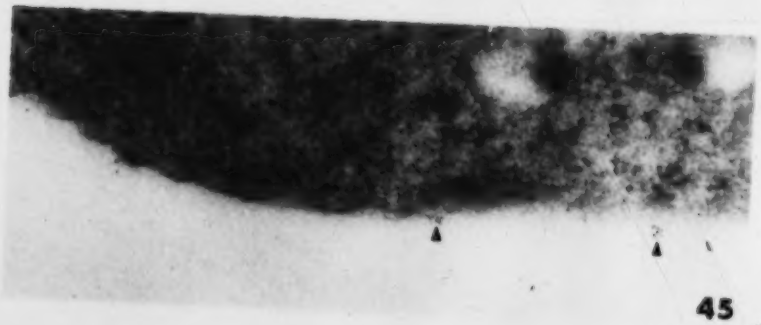

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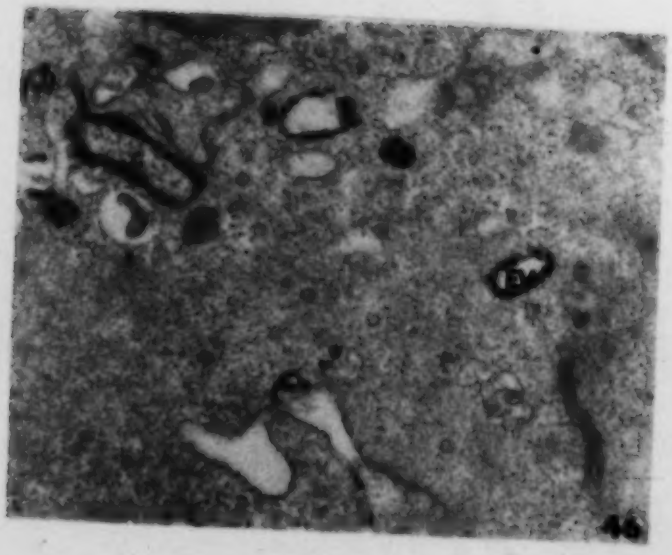

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horseradish peroxidase without any prior conjugation procedure using glutaraldehyde. If the surface-bound lectin could be labelled in this way, alteration in the binding specificity by glutaraldehyde would not be a factor. This means that the natural or native lectin binding sites could be localized. However, the results of this method were inconsistent. When the reaction product was present, it was very dense (Fig. 46), but there was apparent non-specific adsorption to the cell surfaces, resulting in electron dense deposits that were along the LE and the VE. Another major interpretive problem with this method was that the exposure of WGA to unfixed embryos at room temperature resulted in the redistribution of the surface sites. The results showed that there was redistribution and endocytosis of the lectin receptors as reflected by the electron dense deposits within the cytoplasm (Fig. 46) near the VE. Therefore, although some of the surfaces would appear to have very little binding, in actuality the bound lectin may have been endocytosed and would not show up in that particular region. For example, the VE frequently appeared to have the WGA binding removed specifically by the hapten. However, with continued sectioning, the reaction product was seen being endocytosed.

In conclusion, this technique had a degree of non-specificity; that is, either the non-specific binding of WGA or HRP produced the reaction product in the controls. The incorporation of very high dosages of HRP and the reactivity of live tissue enhance this apparent non-specific binding. The technique was considered unsatisfactory due to the presence of reaction product in the control experiments.

E. WGA localized by incubation with glucose oxidase (fixed and unfixed tissue)

The distribution of WGA binding sites may be revealed indirectly with glucose oxidase (GO); that is, when the WGA is inhibited from binding, as in the presence of its hapten inhibitor, GO activity is not revealed at the cell surface (Figs. 47 to 51). The presence of an electron dense deposit would reveal WGA binding sites indirectly by the GO activity (Figs. 52 to 56). The results indicated that all the surfaces of the Stage 1 embryo possess WGA binding sites. The DE and VH appeared to have the highest density of WGA binding sites, as shown by their denser reaction product (Figs. 52 and 56). There is also an

Figs. 47 to 51 Electron micrographs of the cell surfaces of the unfixed Stage 1 chick embryo treated with 50 µg/ml WGA + 0.5 M GlcNAc, rinsed and then incubated with 1 mg/ml GO (to reveal WGA upon the cell surfaces). The embryos were then rinsed and fixed. See Materials and Methods for complete details.

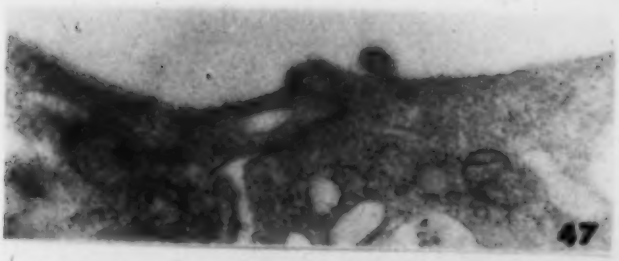
Fig. 47 The dorsal surface of the epiblast, unstained (x 37,100).

Fig. 48 The lateral surface of the epiblast, unstained (x 37,100).

Fig. 49 The ventral surface of the epiblast, unstained (x 37,100).

Fig. 50 The dorsal surface of the hypoblast, unstained (x 37,100).

Fig.51 The ventral surface of the hypoblast, unstained
(x ,100).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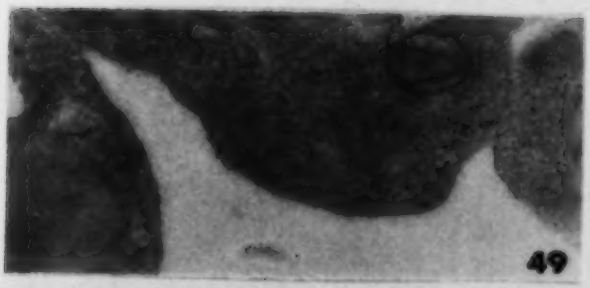
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Figs. 52 to 56 Electron micrographs of the cell surfaces of the unfixed Stage 1 chick embryo treated with 50 µg/ml WGA for 30 minutes at room temperature, rinsed and then incubated with 1 mg/ml GO (to reveal WGA upon the cell surfaces). The embryos were then rinsed again and fixed, see Materials and Methods for complete details. Arrows: indicate reaction product of GO revealing WGA binding sites.

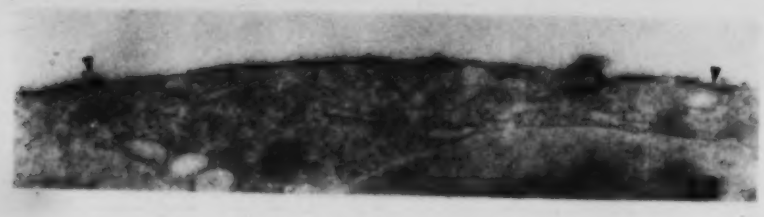
Fig. 52 The dorsal surface of the epiblast, unstained (x 37,100).

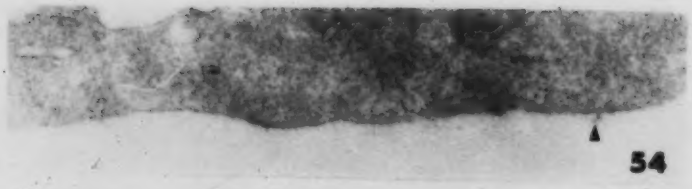
Fig. 53 The lateral surface of the epiblast, unstained (x 37,100).

Fig. 54 The ventral surface of the epiblast, unstained (x 37,100).

Fig. 55 The dorsal surface of the hypoblast, unstained (x 37,100).

Fig. 56 The ventral surface of the hypoblast, unstained (x 37,100).





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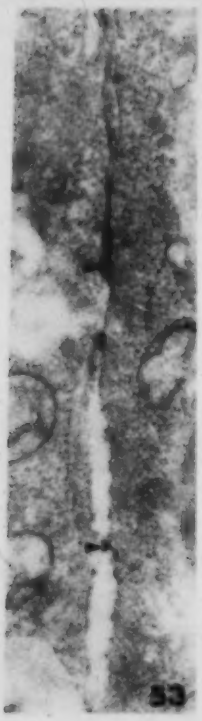

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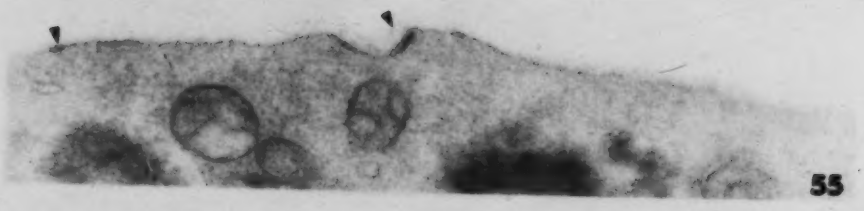

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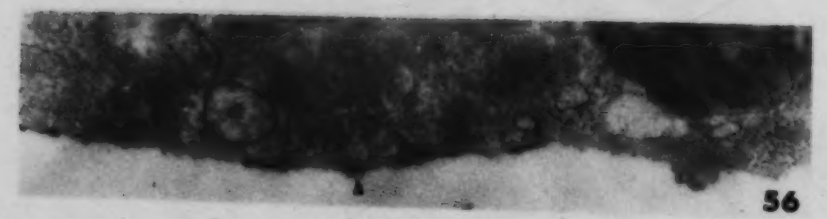

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apparent redistribution of WGA binding sites as revealed by the patching or clumping of the reaction product on the DE and VH (Figs. 52 and 56). The patching of WGA receptors produces localized concentrations of GO and increased reaction product in these regions. In addition, the density of the reaction produce could possibly be enhanced by using a GO enzyme that has a higher specific activity than the one used here.

In an effort to study the natural state of the WGA receptor sites, the method was modified as follows: The embryos were briefly fixed with 2.5% glutaraldehyde for 10 minutes. This prefixation stabilized not only the surface glycoproteins but most of the cellular organelles as well. Furthermore, cell shape is retained, so that positive identification of cell type is enhanced. All these characteristics are valuable when the in situ state is being examined. This brief fixation only inhibited the redistibution of the WGA binding sites and not the apparent density of the reaction product. In the controls, the specific hapten GlcNAC inhibited all binding of WGA so that no electron dense deposit was visualized on any cell surface. When WGA was allowed to react with the embryo in the absence of the hapten, followed by GO, the distribution

of the WGA binding sites was revealed (Figs. 57 to 61). Once again, the WGA binding sites are located upon all the surfaces, indicating that all surfaces possess exposed GlcNAc residues.

In conclusion, this is a specific method of localizing WGA binding sites upon unincubated chick embryos. Furthermore, the distribution of GlcNAc residues as revealed by the GO labelling is the same as that determined by the WGA-HRP conjugation method with 0.01% glutaraldehyde; that is, all surfaces of the Stage 1 embryo possess terminal GlcNAc residues, with the DE and VH having a higher density than the other surfaces.

Figs. 57 to 61 Electron micrographs of the cell surfaces of the prefixed Stage 1 chick embryo treated with 50 µg/ml WGA, rinsed and then incubated with 1 mg/ml GO (to reveal the WGA binding sites). See Materials and Methods for complete procedure. Arrows indicate GO reaction product revealing WGA binding sites.

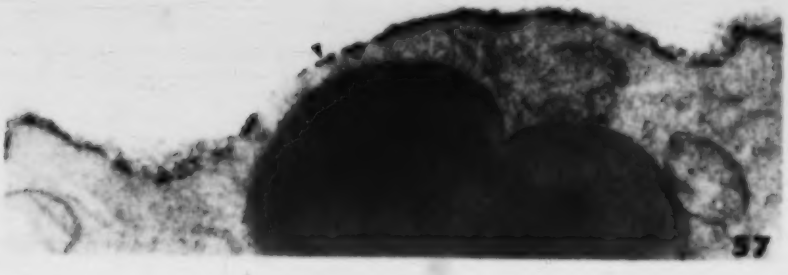
Fig. 57 The dorsal surface of the epiblast, unstained (x 37,100).

Fig. 58 The lateral surface of the epiblast, unstained (x 37,100).

Fig. 59 The ventral surface of the epiblast, unstained (x 37,100).

Fig. 60 The dorsal surface of the hypoblast, unstained (x 37,100).

Fig. 61 The ventral surface of the hypoblast, unstained (x 37,100).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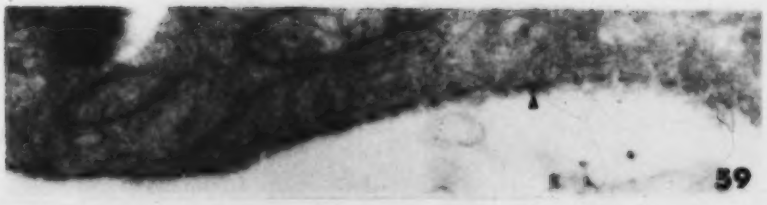
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2. Stage 5 Embryos

Due to the previous work, only two methods were used to determine the distribution of the WGA binding sites upon the Stage 5 embryo. WGA was conjugated to HRP with 0.01% glutaraldehyde to directly label the binding site and the indirect method of exposing the distribution of WGA binding sites by the activity of GO.

A. WGA-HRP conjugated with 0.01% glutaraldehyde (fixed tissue)

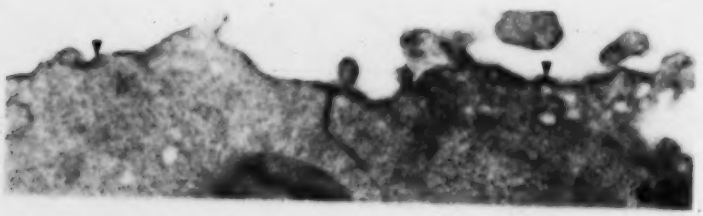
In Stage 1 embryos, conjugation of HRP to WGA with 0.01% glutaraldehyde resulted in a molecule that bound specifically to GlcNAc residues on the cell surface; that is, control experiments using GlcNAc as the hapten inhibitor showed no conjugate binding. By contrast, in the Stage 5 embryos, controls performed in exactly the same manner always showed conjugate binding, i.e. the WGA bound to the DE, the ventral surfaces of the mesenchyme (VM) and dorsal and ventral endoblast (DN and VN) in the presence of GlcNAc (Figs. 62 to 64). Furthermore, it is shown that the binding of the conjugate had a similar distribution with or without the hapten. It may be concluded from this that the

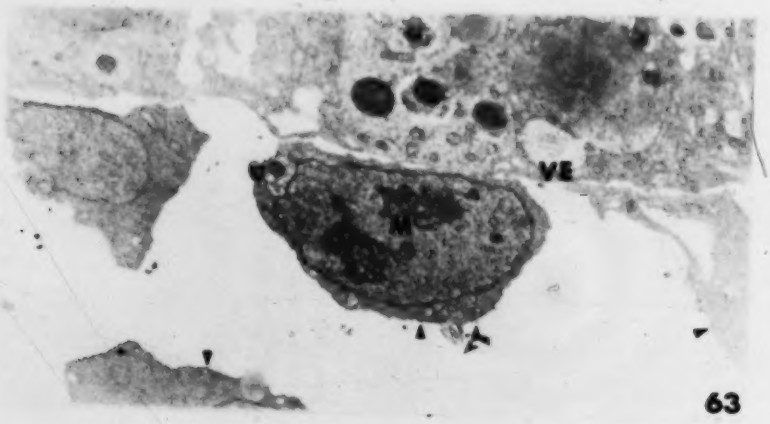
Figs. 62 to 64 Electron micrographs of the cell surfaces of the prefixed Stage 5 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.01% glutaraldehyde) + 0.5 M GlcNAc for 30 minutes at room temperature and then rinsed. Arrows indicate WGA-HRP reaction product.

Fig. 62 The dorsal surface of the epiblast (lateral to the primitive streak), unstained (x 18,150).

Fig. 63 The ventral surface of the epiblast (lateral to the primitive streak) and mesenchyme cells, unstained (x 4,620) M-mesenchyme cells, VE-ventral surface of epiblast.

Fig. 64 The surfaces of mesenchyme cells close to the endoblast cells, lateral to the streak, unstained (x 8,085) M-mesenchyme cell, N-endoblast cell, DN-dorsal surface of endoblast.





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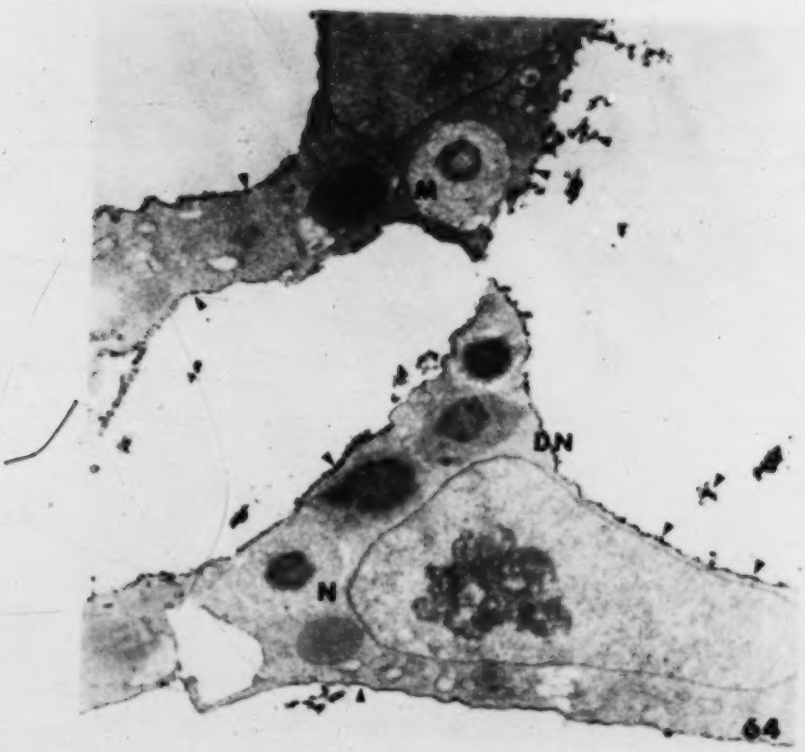

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hapten is not binding WGA with as great an affinity as the WGA is binding to the cell surface. This will be discussed later.

The binding of the WGA-HRP conjugate to the cell surfaces of the Stage 5 embryo had an apparent consistent distribution, although not hapten removable. The DE had WGA binding sites lateral to the primitive streak and within the streak (Figs. 65 and 69 respectively). However the mesenchyme cells showed a definite pattern in their distribution of WGA binding sites. The mesenchyme cells that were near to the primitive streak or the VE, had a very low density of the conjugate, while the mesenchyme cells that were further away from the primitive streak or closer to the endoblast had a higher density of the conjugate (Figs. 66 to 68). It appeared that as the mesenchyme cells migrated away from the streak, the dorsal surfaces of the mesenchyme (DM) possessed fewer binding sites than the ventral surfaces of the mesenchyme cells (VM) (Figs. 71 and 72). Also, the dorsal surface of the endoblast (DN) had a greater density of WGA-HRP binding sites than the ventral surfaces of the endoblast (VN) (Figs. 68 and 73). Finally, only the LE and VE failed to bind any conjugate, while all other cell surfaces had a definite reaction product upon them (Figs. 69 to 73).

Figs. 65 to 66 Electron micrographs of the prefixed Stage 5 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.01% glutaraldehyde) for 30 minutes at room temperature then rinsed. Arrows indicate WGA-HRP reaction product.

Fig. 65 The dorsal surface of the epiblast, lateral to the primitive streak, unstained (x 4,062).

Fig. 66 The ventral surface of the epiblast and mesenchyme cells, lateral to the primitive streak, unstained (x 4,062) M-mesenchyme cell=s, E-epiblast cell. Arrows indicate WGA-HRP reaction product.

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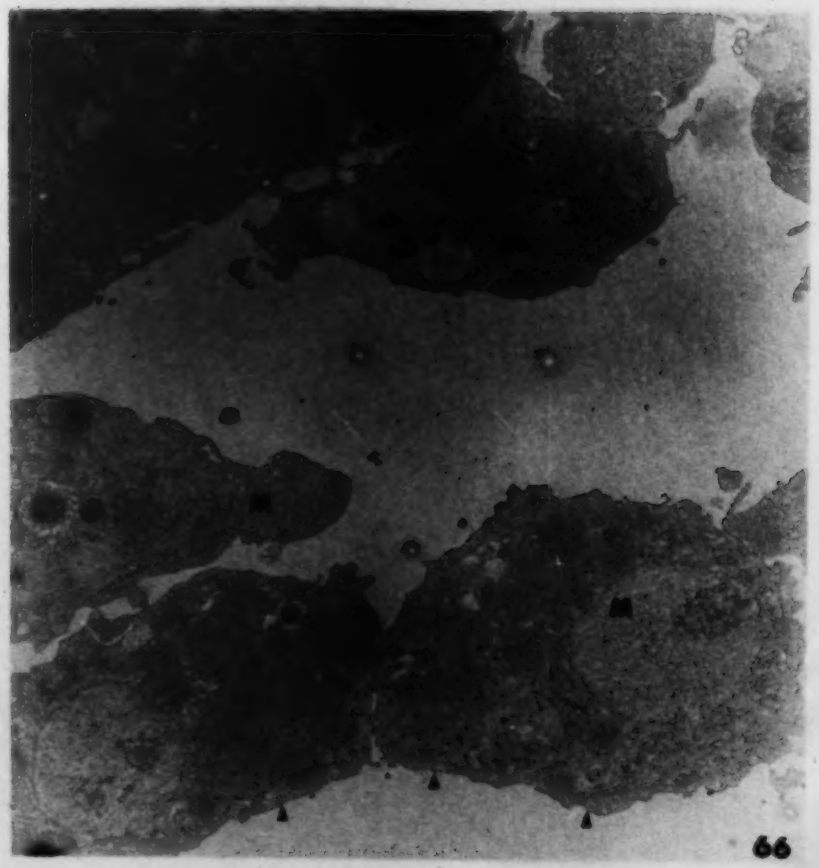

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Figs. 67 and 68 Electron micrographs of the prefixed Stage 5 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.01% glutaraldehyde) for 30 minutes and then rinsed. Arrows indicate WGA-HRP reaction product.

Fig. 67 The ventral surface of the epiblast, lateral to the primitive streak, unstained (x 8,085). E-epiblast cell, BL-basal lamina, M-mesenchyme cell.

Fig. 68 The endoblast cell lateral to the primitive streak, unstained (x 8,085) M-mesenchyme cell, DN-dorsal endoblast, N-endoblast cell.

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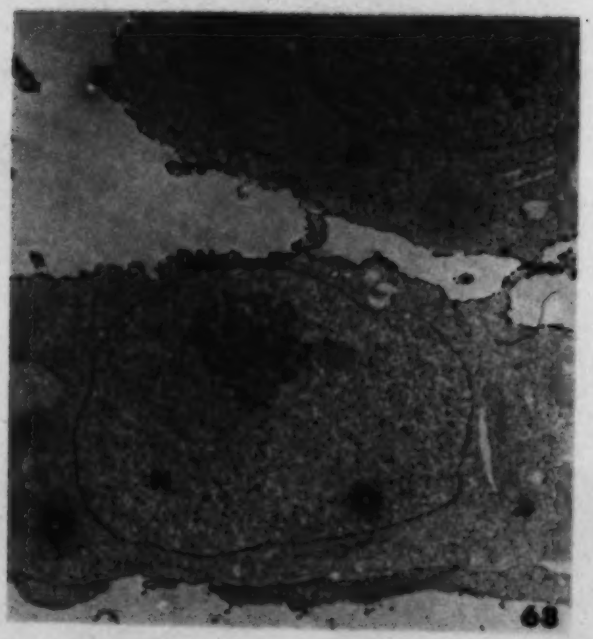

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Figs. 69 to 73 Electron micrographs of the cell surfaces of the prefixed Stage 5 chick embryo treated with 50 µg/ml WGA-HRP (conjugated with 0.01% glutaraldehyde) for 30 minutes at room temperature and then rinsed.

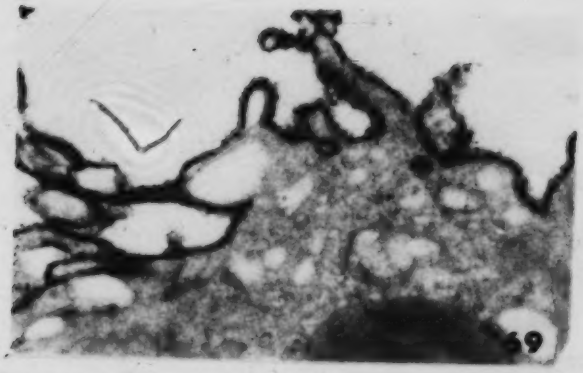
Fig. 69 The dorsal surface of the epiblast within the primitive streak, unstained (x 37,100).

Fig. 70 The lateral surface of the epiblast, unstained (x 37,100).

Fig. 71 The dorsal surface of the mesenchyme cells away from the primitive streak, unstained (x 37,100).

Fig. 72 The ventral surface of the mesenchyme cells away from the primitive streak, unstained (x 37,100)

Fig. 73 A hypoblast cell away from the primitive streak, unstained, (x 18,150). M-mesenchyme cell, DN-dorsal surface of endoblast, N-endoblast cell.



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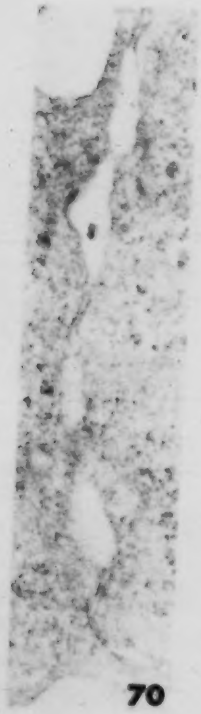

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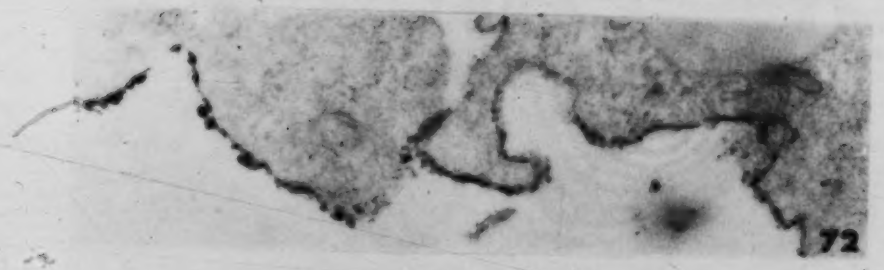

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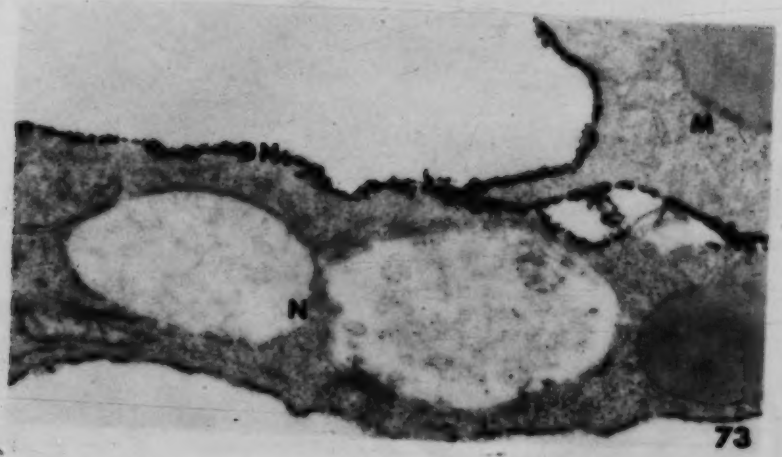


73

In conclusion, although this binding is not entirely removeable by GlcNAc alone, this method still appears to be reflecting a binding site with a very high attraction for the lectin conjugate. Purely non-specific binding might be expected to occur on various cell surfaces at random instead of consistently on the DE, VM, DN and VN. However, due to the failure of the hapten to remove the lectin conjugate, little may be concluded about the structure of the binding site.

B. WGA localized by incubation with glucose oxidase (fixed tissue)

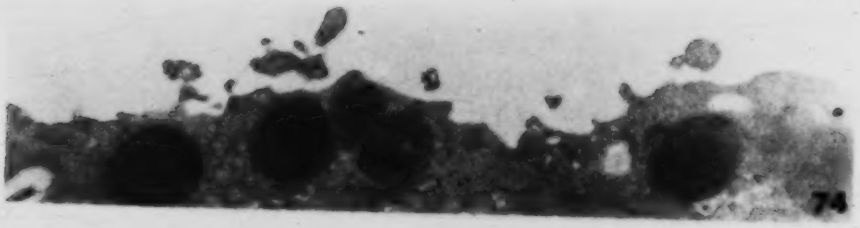
The final ten figures have been arranged so that the distribution of WGA binding sites on Stage 5 embryos may be examined. This method of indirectly labelling the lectin has proven to be the most specific and reliable. The hapten controls show that all binding has been abolished (Figs. 74 to 78). It should be noted that the DE, VM and DN show no binding in the presence of the hapten GlcNAc. Also, the VE with its prominent extracellular material (basement membrane) does not non-specifically adsorb or bind the WGA molecule (Fig. 77).

Figs. 74 to 76 Electron micrographs of the cell surfaces of the prefixed Stage 5 chick embryo treated with 50 µg/ml WGA plus 0.5 M GlcNAc for 30 minutes, then rinsed and incubated with 1 mg/ml GO (to reveal the WGA) See Materials and Methods for complete details.

Fig. 74 The dorsal surface of epiblast, unstained (x 9,900).

Fig. 75 The ventral surface of the epiblast and mesenchyme cells, unstained (x 9.900). E-epiblast cell, M-mesenchyme cell, arrows indicate basal lamina.

Fig. 76 The endoblast and ventral surface of mesenchyme cells, unstained (x 9,900). M-mesenchyme cell, N-endoblast cell, DN-dorsal surface of endoblast.



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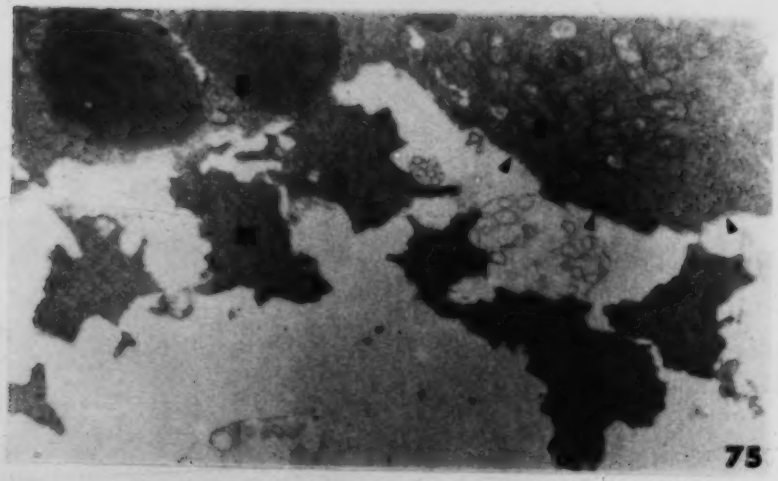

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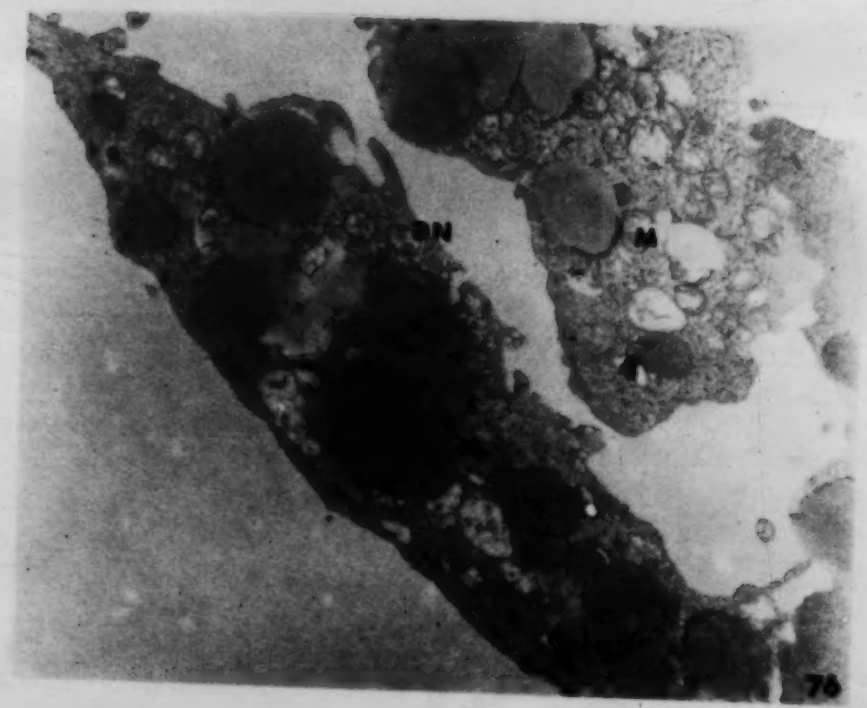

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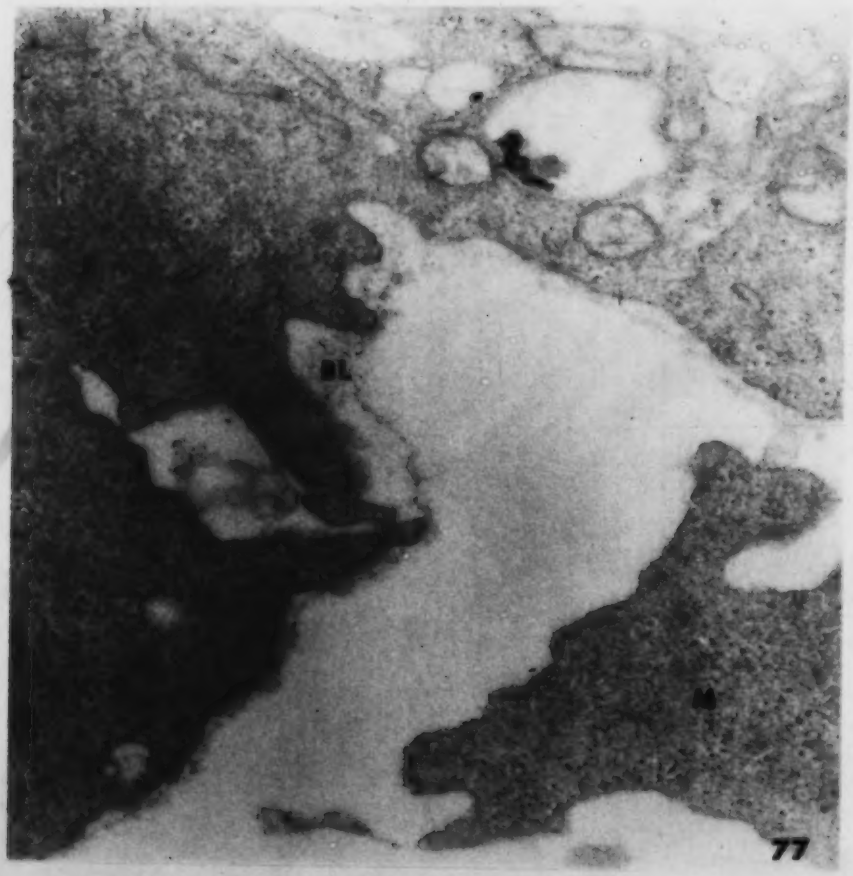
Figs. 77 and 78 Electron micrographs of the cell surfaces of the prefixed Stage 5 chick embryo treated with 50 μg/ml WGA plus 0.5 M GlcNAc for 30 minutes at room temperature, rinsed, and then 1 mg/ml GO (to reveal WGA binding). See Materials and Methods for complete details.

Fig. 77 The ventral epiblast, unstained (x 37,100) Bl-basal lamina, E-epiblast cell, M-mesenchyme cell.

Fig. 78 The endoblast, unstained (x 9,900) DN-dorsal surface of the endoblast.



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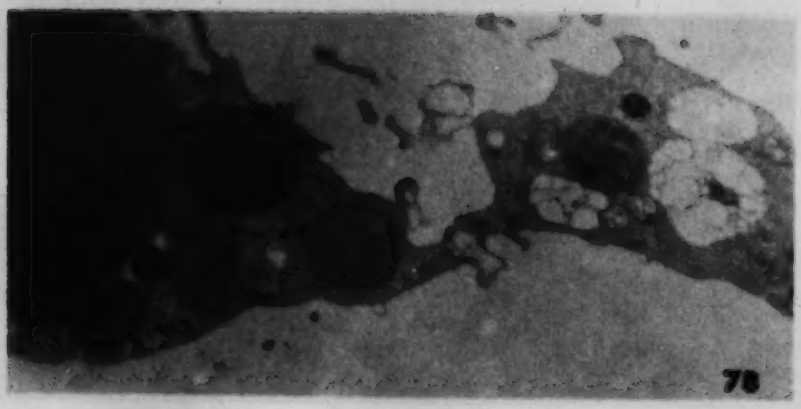


78

Sections were taken from two areas: through the streak and lateral to the streak. The results will show that the surfaces on which the mesenchyme migrates, especially the DN, have a greater density of exposed GlcNAc residues.

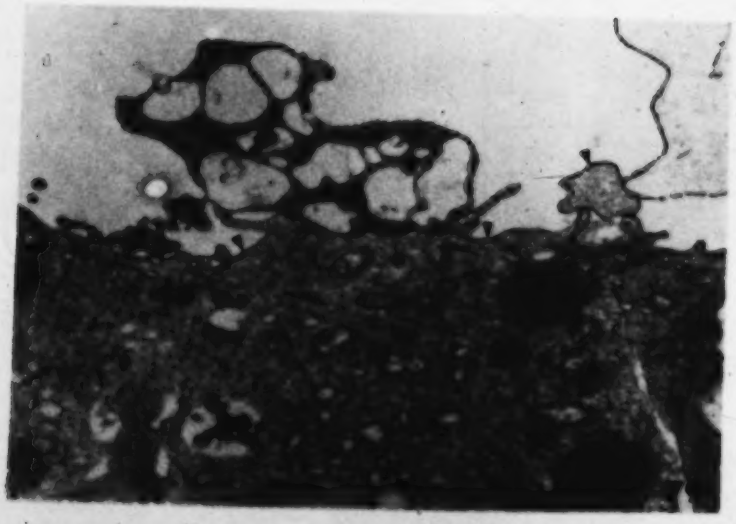
Through the primitive streak, WGA binding sites were located upon the DE, the ventral surfaces of the mesenchyme cells which are in close proximity with the endoblast and the DN (Figs. 79 to 82). Just lateral to the streak, the binding is enhanced upon the VM and DN (Figs. 81 and 82), with the appearance of slight reaction product on the VE (Fig. 88). The DE appears to have an affinity for the WGA whether it is near the streak or lateral to it (Figs. 83 and 84 respectively), and the LE has no affinity for WGA (Fig. 87).

It appears that as cells pass through the streak they losetheir WGA binding sites. This is reflected by the failure of the GO to reveal any WGA bound to the surfaces of cells within the streak (Figs. 80, 85 and 86). However, as the mesenchyme migrates downwards and laterally from the streak, the binding sites for WGA reappear (Figs. 81, 82 and 89). Also, WGA has an affinity for the VE only lateral to

Figs. 79 to 94 Electron micrographs of the cell surfaces of the prefixed Stage 5 embryo treated with 50 μg/ml WGA for 30 minutes at room temperature, then rinsed and incubated with 1 mg/ml GO (to reveal the WGA binding sites). Complete details in the Materials and Methods.

Fig. 79 The dorsal surface of the epiblast in the primitive streak, unstained (x 9,900). Arrows indicate WGA-GO reaction product.

Fig. 80 The ventral surface of the epiblast in the primitive streak, also the dorsal surface of the mesenchyme, unstained (x 9,900). E-epiblast cell, M-mesenchyme cell.



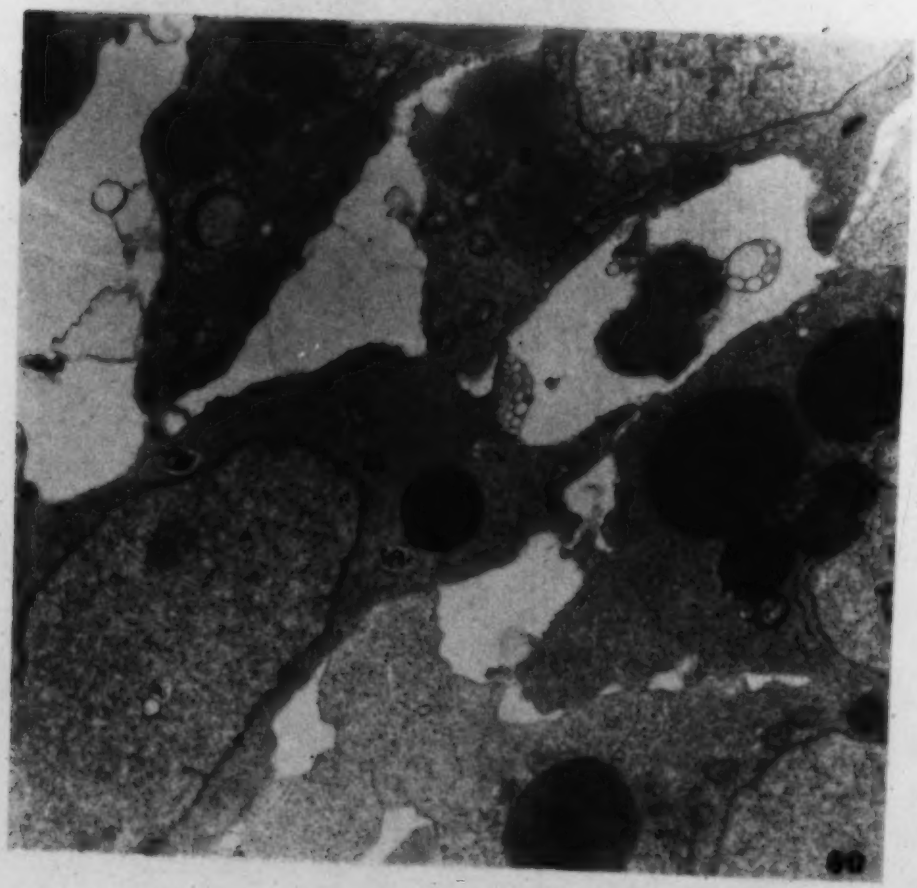
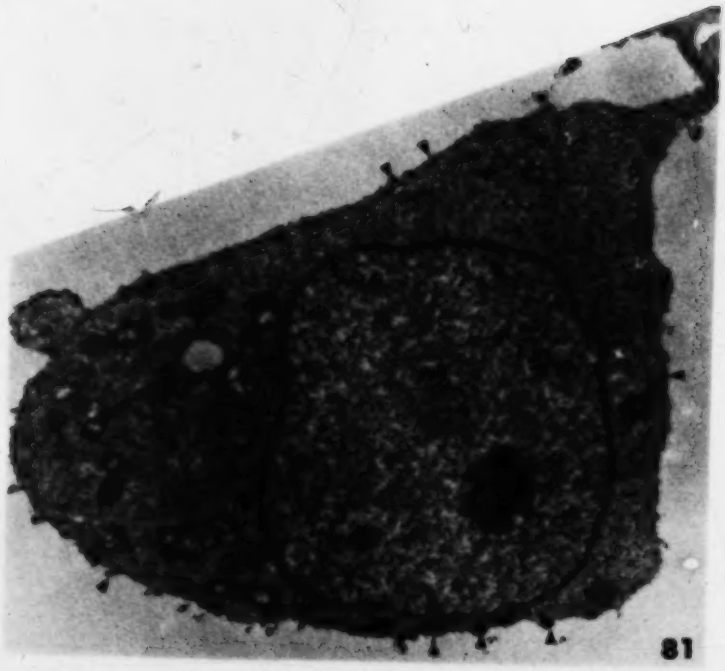


Fig. 81 Mesenchyme cell away from the primitive streak, unstained, (x 9,900). Arrows indicate WGA--GO reaction product (Note the polarity).

Fig. 82 Mesenchyme cell in contact with the endoblast cell, unstained (x 9,900). M-mesenchyme cell, N-endoblast cell. Arrows indicate WGA--GO reaction product (Note little binding on VN) VN-ventral surface of the endoblast.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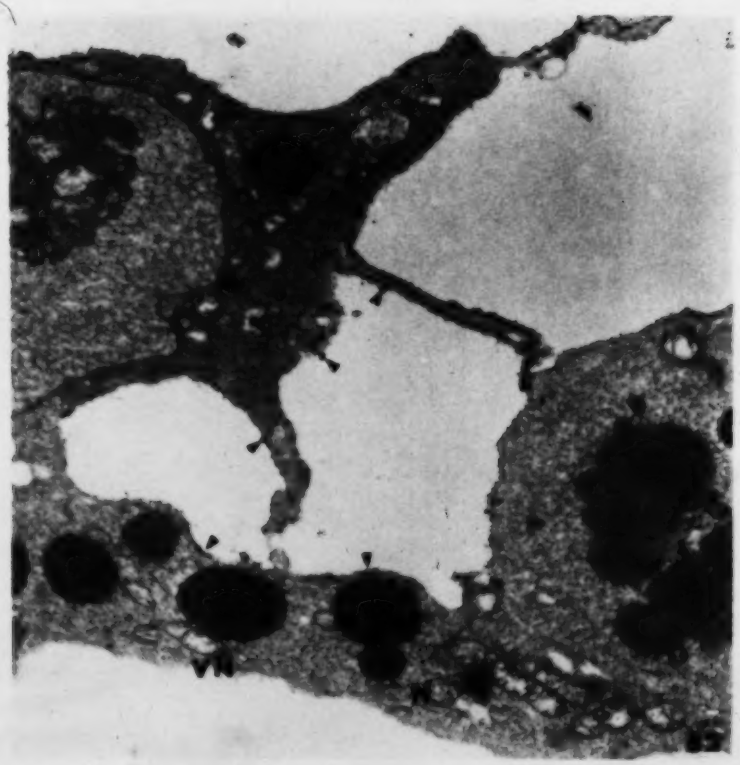
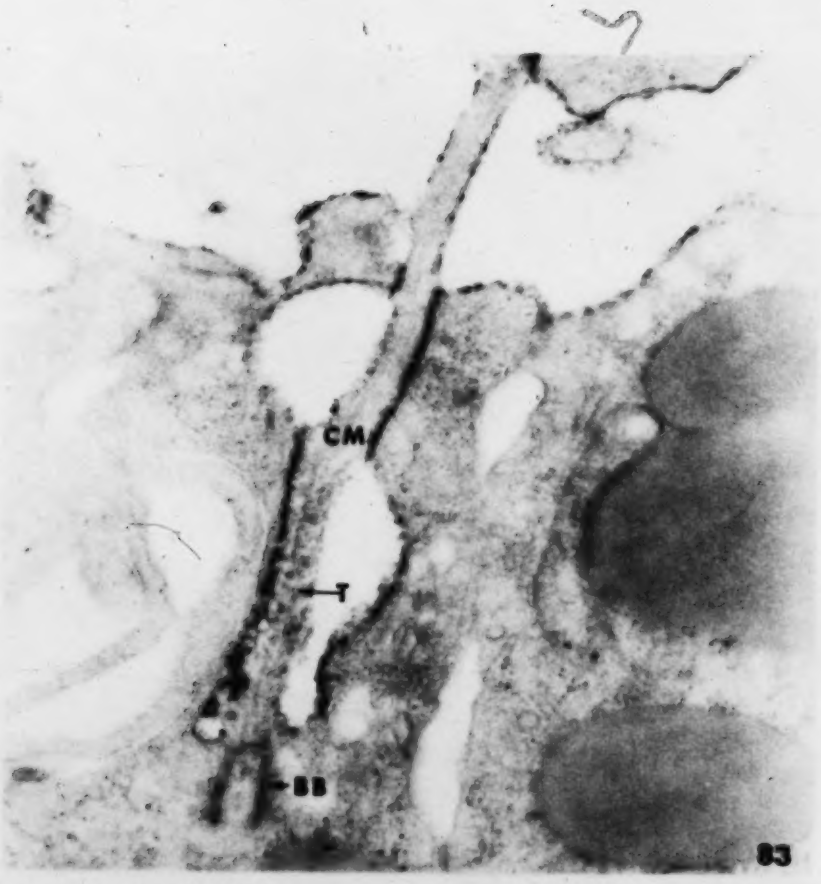

VM
N
82

Fig. 83 Dorsal epiblast in the primitive streak, unstained (x 37,100). CM-cilium, BB-basal body and T-tubules.

Fig. 84 Dorsal epiblast lateral to the primitive streak, unstained (x 37,100).


CM
T
BB
83

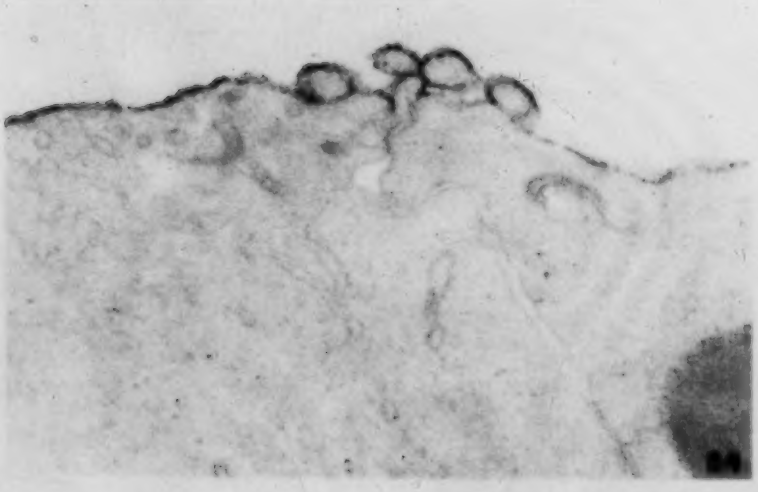

84

Fig. 85 The lateral surfaces of the cells invaginating through the primitive streak, micrograph closer to the dorsal surface of the epiblast, unstained (x 37,100). Note: no visible WGA--GO reaction product.

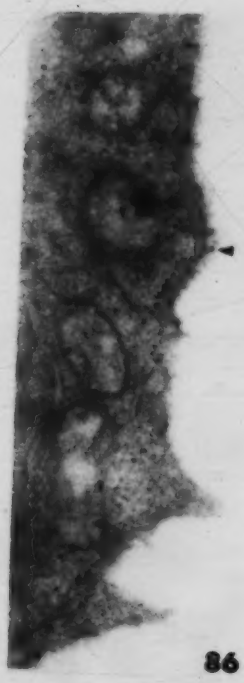
Fig. 86 The lateral surface of a cell invaginating through the primitive streak, micrograph from the ventral portion of the cell, unstained (x 37,100).
Arrow indicates slight WGA--GO reaction product.

Fig. 87 The lateral surface of the epiblast, unstained (x 37,100).

Fig. 88 The ventral surface of the epiblast, unstained (x 37,100). Arrows indicate WGA--GO reaction product on basal lamina.

Fig. 89 The ventral surface of the mesenchyme which is in close proximity to the endoblast, unstained (x 37,100). Arrows indicate slight WGA--GO reaction product on basement membrane-like material (amorphous deposit).

85



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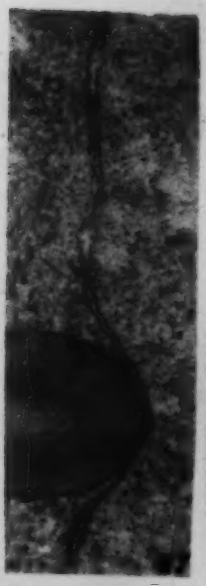

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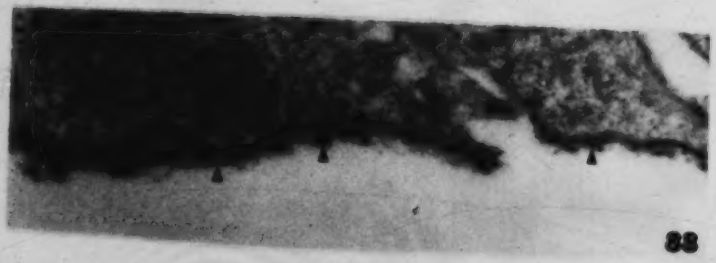

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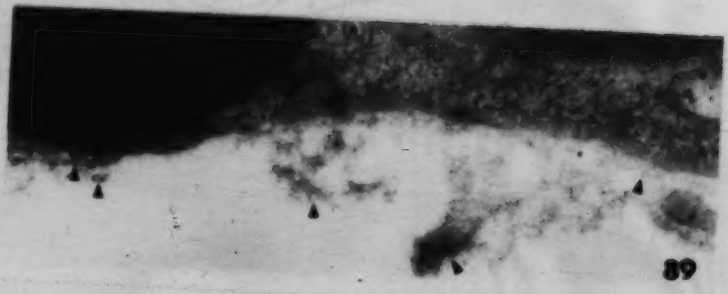


89

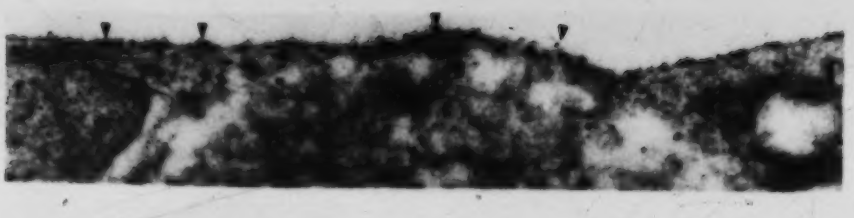
the primitive streak (Fig. 88) and not within the streak (Fig. 80). Finally, the endoblast appears to have a relatively consistent distribution of WGA binding sites, these always being present dorsally and not ventrally. Beneath the streak, the DN binds WGA while the VN does not (Figs. 90 and 91). Lateral to the streak, the DN has a greater number of WGA binding sites than the VN (Fig. 92).

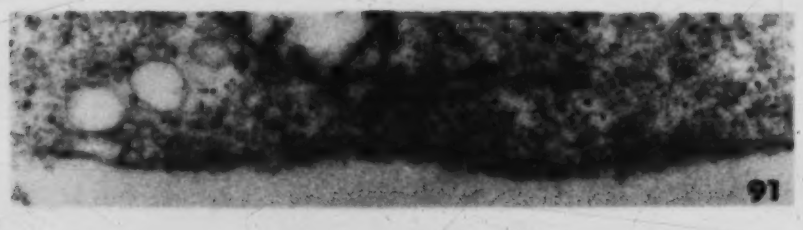
The thick basement membrane of the VE (Fig. 77) has a slight affinity for WGA (Fig. 88), indicating some exposed GlcNAc residues. The appearance of a thick extracellular substance on the mesenchyme near the endoblast cells was unexpected since it is not visualized in the absence of the lectin and yet appears similar to the basement membrane associated with the VE, (Fig. 89). The reaction product upon these surfaces, the VE, the VM and the DN, all appear as a fluffy, patchy, electron dense deposit. Furthermore, the density of the reaction product increases upon the VE and DN further away from the primitive streak. It is possible that the surface carbohydrate groups are arranged so that the glycoproteins lining the cavity where the mesoderm is actively migrating possess exposed GlcNAc residues. Also, these glycoproteins appear to extend some distance from the cell surfaces, perhaps as a network upon

Fig. 90 The dorsal surface of the endoblast directly beneath the primitive streak, unstained (x 37,100). Arrows indicate WGA--GO reaction product.

Fig. 91 The ventral surface of the endoblast directly beneath the primitive streak, unstained (x 37,100). Note: this surface does not bind WGA.

Fig. 92 The endoblast cells lateral to the primitive streak, unstained (x 37,100). Arrows indicate WGA-GO reaction product. Note: the polarity in the distribution DN-dorsal endoblast.

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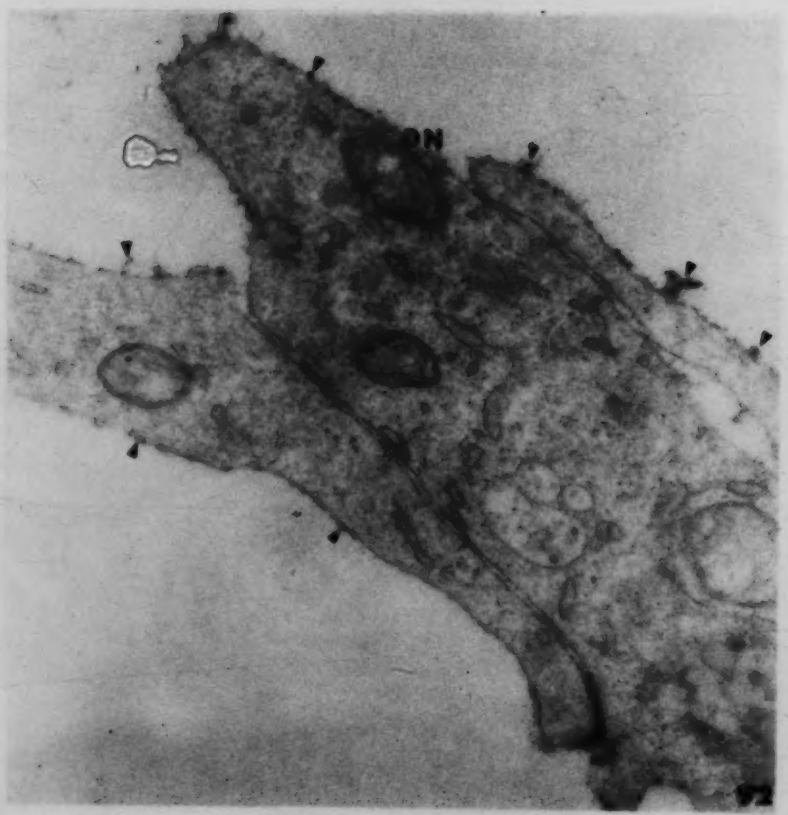

N
92

which the mesenchyme can migrate. The mesenchyme cells appear to be actively producing a meshwork of extracellular substances, like the VE, which appears to be greatest as the cells approach the endoblast. Perhaps the mesenchyme cells are actively producing a substrate for their migration.

A schematic representation of the distribution of WGA binding sites, as revealed with glucose oxidase on Stage 1 and Stage 5 embryos, is presented in Figures 93 and 94. There has been a change in the distribution of the lectin receptor sites, which suggests a polarity of the terminal N-acetyl-glucosamine residues in the Stage 5 embryo. It also appears that only the indirect labelling technique of WGA by glucose oxidase is effective in defining the <u>in situ</u> distribution of WGA binding sites on the chick embryo cell surface.

Fig. 93 A schematic representation of a Stage 1 chick embryo showing the distribution of WGA binding sites. E-epiblast, H-hypoblast.

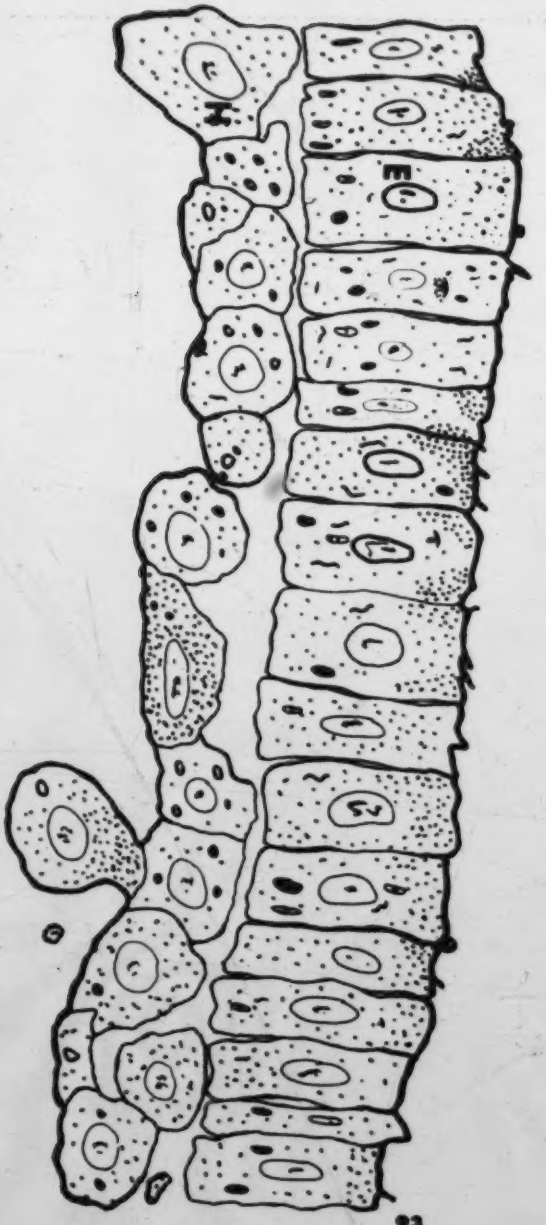
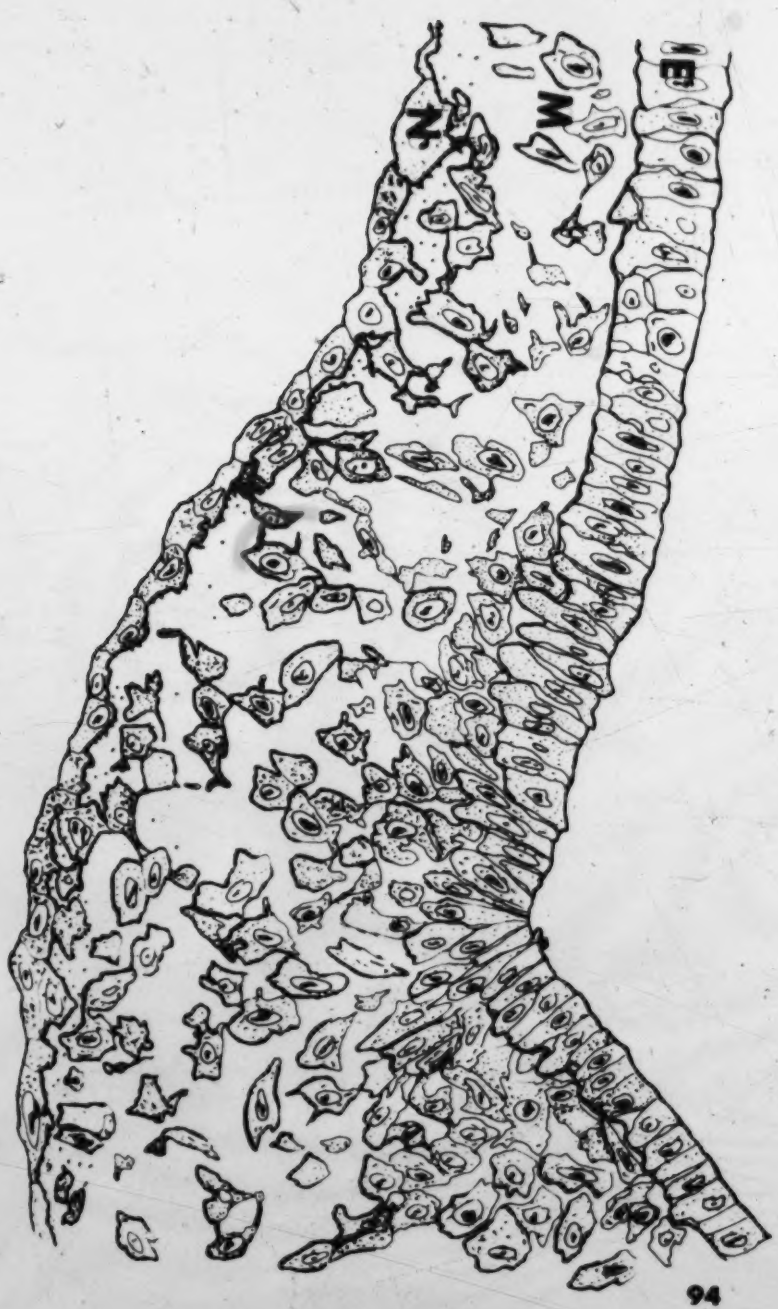

M
E
93

Fig. 94 A schematic representation of a State 5 chick embryo showing the distribution of WGA binding sites. E-epiblast, M-mesenchyme and N-endoblast.


N
W
E
94

DISCUSSION

The cell surface has been implicated as the mediator of cellular events such as adhesion, recognition, communication, locomotion and growth regulation (Cook and Stoddart, 1973; Moscona, 1974; Poste and Nicolson, 1976b). Upon the discovery that cells had a coating of complex oligosaccharides (reviewed by Martinez-Palomo, 1970), the molecular basis for the cell surface involvement in cellular behavior was established (Roseman, 1975). In studying the involvement of the cell surface carbohydrates in gastrulation, the distribution of WGA binding sites was examined on Stage 1 and Stage 5 chick embryos.

The gastrulating chick embryo provides an excellent model for the involvement of the cell surface in altered cellular behavior. This early development results in the appearance of three cell types from the original two cell types. The first cells (endoblast) to invaginate through the primitive streak are invasive, the second cell type that passes through the streak is migratory (mesoderm) and the cells that do not invaginate are sedentary (epiblast). In

addition, the chick embryo is very easy to obtain and prepare for ultrastructural visualization in the electron microscope.

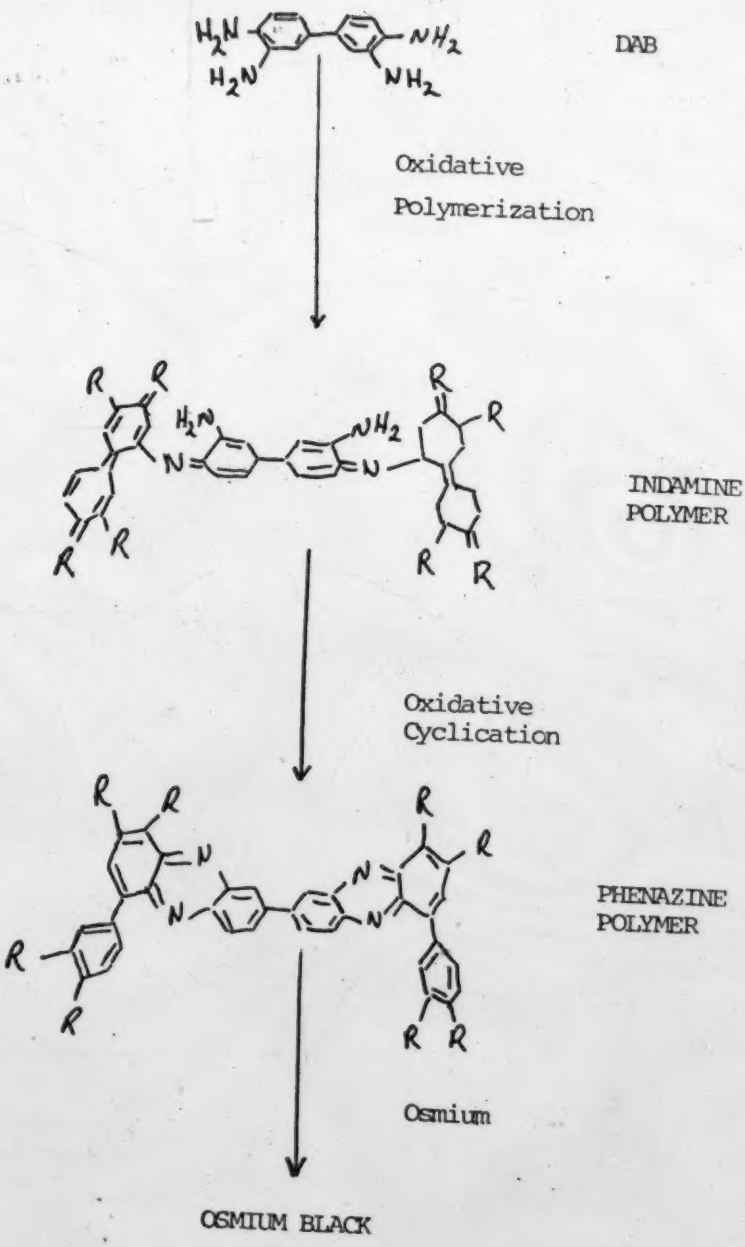
The morphogenetic movements that occur within the initial 20 hours of development may be reflected by altered cell surface characteristics. It was demonstrated that there was a redistribution of WGA binding sites on the cell surfaces during this early development. This suggests that the distributions of the cell surface glycoproteins, glycolipids or glycosaminoglycans, which are specifically labelled with WGA, may be involved in the morphogenetic movements that occur during gastrulation of the chick embryo.

The lectin WGA was exposed upon the surface of the chick embryo by a variety of techniques. However, the indirect labelling technique with the enzyme marker glucose oxidase (GO) resulted in a consistent, hapten removeable tagging of GlcNAc residues (Francois and Mongiat, 1977). The only other technique that may be correlated to these results was the direct labelling technique of cross-linking WGA to the enzyme marker horseradish peroxidase (HRP) with 0.01% glutaraldehyde (Huet and Garrido, 1972). Unfortun-

ately, two other promising techniques have recently been reported but were not employed in the present study: the indirect labelling technique of WGA with an ovomucoid-colloidal gold marker (Geoghegan and Ackerman, 1977) and the tagging of WGA with glycosylated HRP or glycosylated ferritin (Kieda et al, 1977).

The enzyme HRP spontaneously combines with WGA and the complex is stabilized by covalent bonds with glutaraldehyde (Huet and Garrido, 1972). This conjugate will bind, via the WGA, to exposed GlcNAc residues and still catalyze, via the HRP molecule, the oxidative polymerization of DAB by hydrogen peroxide. The oxidation of DAB forms indamine polymers followed by further quinoid addition to the primary amine resulting in oxidative cyclization to a phenazine polymer (see following page, Seligman et al, 1968). The final polymer is a brown precipitate and is osmiophilic so it becomes electron dense.

The enzyme GO has exposed GlcNAc residues so it will bind specifically to WGA molecules upon the cell surface. This specific binding is then stabilized with covalent bonds by glutaraldehyde fixation (Francois and Mongiat, 1977). The enzyme action of GO is to cleave glucose into hydrogen peroxide and gluconic acid. The free hydrogen peroxide oxidizes DAB. The HRP in the reaction



(Seligman et. al., 1968)

mixture catalyzes the oxidative polymerization at the site of hydrogen peroxide production, which is at the GO molecule. Therefore the brown precipitate or the electron dense osmium-black reaction product will occur only in conjunction with the GO molecule.

One of the major concerns of ultrasctructural visualization of lectin binding sites is the accessibility of the electron dense marker to all possible sites (Temmink et al, 1975). The relative size of the lectin, the marker employed, or the conjugated lectin marker, will determine the ability of these molecules to penetrate efficiently to all the possible binding sites. For example, the ovomucoid-colloidal gold marker could not penetrate through junctional complexes, so this technique may only be useful for dissociated cell systems. The present study employed two techniques that reflected binding on all cell surfaces on Stage 1 embryos and excellent penetration to the ventral surface of the epiblast on the Stage 5 embryos. It is assumed that both the WGA-HRP and WGA-GO binding patterns reflect the distribution of all the possible WGA binding sites on both Stage 1 and Stage 5 embryos.

Another concern is to ensure that the binding patterns of WGA reflected by the electron dense deposit are in their natural state. It is essential that the distribution of terminal GlcNAc residues has not been altered by the methods employed in preparing the tissue for observation in the electron microscope. For this reason, the embryos were sometimes briefly fixed with glutaraldehyde before experimental manipulation. The action of the glutaraldehyde is believed to be the cross-linking of the proteins in the membranes by forming methylene bridges between available amino groups (Gersch, 1959; Sabatini et al, 1963; Pease, 1964) and the stabilizing of the protein distribution. The brief exposure of tissue to glutaraldehyde causes membranes to become stronger and more rigid but also allows for some degree of continued enzyme function. This was the method employed in the fractionation of Golgi bodies and subsequent analysis of purity by the marker enzyme acid phosphatase (Fleischer et al, 1969). Therefore, it may be assumed that the glutaraldehyde fixation is not altering the nature of the distribution of the WGA binding site. Evidence supporting the validity of this assumption is that the comparison between binding patterns of unfixed versus fixed tissues resulted in relatively similar overall distributions.

The failure of the simple hapten to inhibit the binding of the WGA-HRP conjugate was a major source of difficulty in the interpretation of the results. Experiments were employed to determine what the substrate was for the non-specific binding of the lectin conjugate. The enzyme pretreatment was not conclusive in demonstrating that this non-specific binding was to glycoproteins or glycosaminoglycans. However, by lowering the final concentration of glutaraldehyde (0.03% to 0.01%) in the cross-linking reaction mixture, the reaction of the WGA-HRP conjugate in the Stage 1 embryo appeared to be specific. Unfortunately, the binding of the similar conjugate to the Stage 5 embryo was not GlcNAc-specific. This raised serious doubts about the technique of coupling WGA to HRP with glutaraldehyde. Whitaker (1976) tested the effect of glutaraldehyde cross-linking upon the antigenicity of bovine myelin encephalitogenic protein. He determined that the two-step coupling method was better at retaining the antigenicity of the conjugate than the one-step method (Avrameas, 1969). Basically, the two-step method differs from the one-step method (which is employed in this study) in that the marker enzyme is activated with glutaraldehyde before the reaction with the antibody (in this case the lectin, WGA). However, he also found that conjugates

prepared by both techniques did not have altered immunogenicity.

The inconsistency in the results of the binding of WGA-HRP (0.01% glutaraldehyde) from the Stage 1 to the Stage 5 embryo may also be the result of the appearance of a substrate with a higher attraction for the conjugate than the simple hapten GlcNAc. It has been demonstrated that the binding site of the WGA has a higher affinity for oligosaccharides like $(GlcNAc)_2$ or $(GlcNAc)_3$ than the monosaccharide GlcNAc (Allen et al, 1973; Privat et al, 1974; Goldstein et al, 1975; Privat and Monsigny, 1975). Therefore, the appearance of a certain oligosaccharide at Stage 5 may have influenced the binding pattern of the WGA-HRP. A possible candidate for the appearance of a new substrate may be hyaluronic acid (HA). Manasek (1975) and Solursh (1976) showed that the gastrulating chick embryo produces copious quantities of HA. This glycosaminoglycan has the repeating oligosaccharide component GlcNAc-glucuronic acid. The binding of WGA to this substrate may be stronger than to the monosaccharide GlcNAc, used as a hapten.

The binding site of WGA may be inferred to have a terminal GlcNAc residue when binding is hapten removeable.

Therefore, almost certainly one type of binding site may be HA, although glycoproteins and glycolipids containing terminal GlcNAc residues would be labelled too. In an effort to determine the amount of binding occurring with HA, Stage 5 embryos were pretreated with hyaluronidase (testicular, Grade VI). However, the pretreatment caused the cells of the Stage 5 embryo to lose their distinguishing morphology so that orientation was impossible. It would be worthwhile to treat prefixed embryos with hyaluronidase so that the enzyme's effect upon the natural WGA binding sites could be interpreted.

It is interesting that the relative distribution of binding of the WGA-HRP conjugate on both Stage 5 and Stage 1 embryos is similar to the distribution of the WGA binding sites as revealed with GO. The binding of the WGA-GO is inhibited by the hapten GlcNAc, so information concerning the distribution of exposed GlcNAc residues may be collected. With WGA-GO labelling, the Stage 1 embryo has an apparent distribution of GlcNAc residues upon all surfaces of the epiblast and hypoblast. Although there appears to be more GlcNAc residues upon the dorsal surface of the epiblast (DE) and ventral surface of the hypoblast (VH), this may be due to an amplification of the reaction product by the

enzyme. With WGA-GO labelling, the Stage 5 embryo has terminal GlcNAc residues on the DE and ventral surface of the epiblast (VE), ventral surface of the mesenchyme (VM), and dorsal surface of the endoblast (DN). Tehre are few exposed GlcNAc residues upon cells that are invaginating through the streak, upon the dorsal surface of the mesenchyme (DM) and the ventral surface of the endoblast (VN). Finally, the lateral surfaces of the epiblast in the Stage 5 embryo do not have any exposed WGA binding sites. These results indicate that the distribution of exposed, terminal GlcNAc residues is altered within these early stages of development. In addition, the cellular differentiation within the Stage 5 embryo appears to be reflected by the cell surface.

The WGA binding site, terminal GlcNAc residues, may be located upon a variety of structures. As was mentioned previously, GlcNAc is a component of HA. This sugar residue may be added to the terminal end of glycoproteins or glycolipids by glycosyltransferases. Since glycolipids are not a common membrane component it may be assumed that the terminal GlcNAc is being located principally upon glycoproteins. Zalik and Cook (1976) partially purified the WGA receptor from 12-day liver cells and found it to be glycoprotein in nature, although the WGA binding site from less differentiated cells has not been isolated.

The intriguing result was that there was revealed in the Stage 5 embryo an amorphous basement membrane-like material upon the DN and VM. This material is presumed to be HA. Manasek (1975) showed with pulse labelling experiments of radioactive GlcNAc that the GlcNAc was incorporated on to all surfaces of the Stage 5 embryo. He also reported the deposition of GlcNAc into HA in the cavity between the epiblast and the endoblast. Fischer and Solursh (1977) confirmed the distributuion of HA in Stage 8 embryos. They found HA associated with the basement membrane of the ectoderm and in association with the mesenchyme. It is interesting to find that there is some WGA binding to the basement membrane of the epiblast on the Stage 5 embryo. The basement membrane, (Low, 1967) also stains non-specifically with Ruthenium red for mucopolysaccharides (Martinez-Palomo, 1970). This suggests that HA may also be associated with the glycosaminoglycans of the basal lamina of the Stage 5 embryo.

The electron dense reaction product found on the VM and DN appears as a fluffy, amorphous deposit. The reaction

product extends some distance from the cell surface and has an appearance similar to the deposit of the basal lamina, although it is not continuous. This specific binding, possibly due to the HA residues, may be identifying the substrate for the migration of the mesenchyme. It has been suggested that the mesenchyme cells use the VE, DN and each other as an aid for their migration (Ebendal, 1976; England and Wakely, 1977) in the Stage 5 embryo. Dodson and Hay (1973) suggested that in the case of mesenchyme migration during formation of the corneal endothelium, the substrate is the basement membrane deposited by the corneal epithelium. Alternatively, it may be that the mesenchyme deposit their own substrate to aid locomotion and migration.

In relation to some recent studies conducted upon chick embryos, the results of the present study are indicative of an alternation of the cell surface with the onset of gastrulation. Dissociated chick embryos up to Stage 5 will aggregate and sort out into two distinct cell populations (Zalik and Sanders, 1974; Sanders and Zalik, 1976). Despite the fact that dissociated Stage 5 embryos are promptly agglutinated with WGA (Zalik and Cook, 1976), the present study has demonstrated that within the embryo

there is a segregation of WGA binding sites onto specific surfaces. The forces responsible for this redistribution may involve the cytoskeleton of the cells, but any definite mechanism cannot be deduced from the present study. The function of the polarity in the WGA binding sites of the Stage 5 embryo may be due to the appearance of new cell types or may be the response of altered cellular behavior.

The differentiation of the cell surface in relation to development has been demonstrated previously. The DE has been shown to have an altered topology from areas within the primitive streak as compared to areas lateral to the streak. Specifically, the cells that are about to invaginate lose their surface microvilli (Bancroft and Bellairs, 1974). Sanders and Zalik (1972) found that anionic sites on the cells that are invaginating were increased. This was revealed with increased lanthanum binding in the primitive streak, and suggested that the cell surface was altered as cells invaginate. Hook and Sanders (1977) found that the patterns of binding of Concanavalin A (Con A) were altered from Stage 1 to Stage 5 embryos. They reported an increased density of Con A binding sites on the DE and the loss of binding on the VE and endoblast. It was thought that the DE had differentiated in preparation for invagination, although

there was no associated change of Con A binding to the cell surface of cells that were invaginating.

The epiblast cells that invaginate through the primitive streak initially have the property of inserting into the sheet of hypoblast cells and displacing them. Rosenquist (1972) called these invasive cells the definitive endoblast and outlined how they form the lower layer of the embryo. Bellairs et al, (1977) used tissue culture experiments and time lapse cinematography to show the behavior of endoblast explants in relation to hypoblast explants. The endoblast cells are very active and invasive; they move as a loose net of cells and eventually are surrounded by the hypoblast. These results suggest that the endoblast have an altered or differentiated behavior which is probably reflected at the cell surface. The endoblast of the Stage 5 embryo have a greater majority of WGA binding sites upon their dorsal surface. Also associated with these cells may be extracellular glycosaminoglycans as revealed only through WGA binding and GO deposition.

The second type of cells that passes through the streak are the mesenchyme. They are migratory and spread laterally between the epiblast and the endoblast. As the

mesenchyme invaginate they constrict dorsally and bulge ventrally, forming a flask-shaped cell (Balinsky and Walther, 1961). The WGA binding sites upon cells that are actively invaginating are very sparse but reappear as the mesenchyme begins its lateral migration. The VM has an increased density of WGA binding sites which has the appearance of an extracellular matrix in places. The extracellular material may be seen spanning two cells - mesenchyme and endoblast - much like the basement membrane of the epiblast.

The presence of the HA within the cavity between the epiblast and the endoblast, as possibly visualized with WGA-GO, may be associated with a number of functions. The HA may be necessary to maintain the water balance and, in essence, the pressure within the cavity (Fischer and Solursh, 1977). It may also be related to the inactivation of adenylate cyclase as suggested by Kelley et al, (1977). This latter study may suggest that the HA is inhibiting the levels of cAMP within the mesenchyme cells resulting in their altered cellular behavior. In addition, the HA may be a substrate that is necessary for the migration of the mesenchyme, which follow the endoblast though the streak but do not invade the endoblast.

The discovery of membrane-bound hemagglutinins from various cellular systems (Rosen et al, 1973; Dysart and Edwards, 1977) has led many investigators to study the possibility of hemagglutinins (lectins) associated with a variety of recognition and variable adhesion systems. Zalik and co-workers (1978, unpublished) have isolated, from the chick embryo, a multivalent protein that caused hapten-specific agglutination of prefixed erythrocytes. This divalent ligand (lectin) appears to be membrane-bound and may be associated with the variable adhesiveness of the cells from a gastrulating chick embryo. These ligands may not only be involved in embryonic systems but may form the basis for tissue-specific adhesion properties. It is thought that plant lectins function in a recognition process between the plant and certain specific beneficial bacteria (Marx, 1977).

The actual function of the noted distribution alteration of terminal GlcNAc residues is open to speculation. To determine the significance, monovalent WGA may be allowed to interact with the chick embryo during gastrulation, and the development monitored. Another line of experimentation may follow the pretreatment of the embryos with specific glycosidases to remove specific cell surface carbohydrates, and the determination of their effect

upon gastrulation. To inhibit the replacement of surface glycosylated proteins, tunicamycin could be incorporated into these experiments (Duksin and Bornstein, 1977). In addition, experiments should be conducted to determine the purpose of the HA upon the gastrulating chick embryo and its involvement with the migrating mesenchyme.

The role of the cell surface in the development of birds and, for that matter, all other organisms is being thoroughly investigated. The complex cellular interactions in morphogenesis and differentiation are being clarified. Within time the information compiled may be applied to specific pathological conditions and have a pratical application.

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